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# An Analysis of Between-Cow Variation in Innate Immunity in Relation to Mastitis Severity

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# AN ANALYSIS OF BETWEEN-COW VARIATION IN INNATE IMMUNITY IN RELATION TO MASTITIS SEVERITY.

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## ABSTRACT

Bovine mastitis remains one of the costliest diseases affecting the dairy industry. Individual susceptibility to mastitis and severity of infection varies between animals and can only be partially explained by genetics. As such, understanding how genetic predisposition coordinately interacts with epigenetic modifications and environmental exposures is necessary to bridge the gap in missing heritability. The role of DNA methylation in regulating the response to bacterial lipopolysaccharide (LPS) was first determined by performing reduced representation bisulfite sequencing on fibroblasts isolated from heifers at 5- and 16-months of age that exhibit an age-dependent up-regulation in LPS-responsiveness. More than 14,000 differentially methylated sites were identified between the two sets of cultures with a trend towards decreased methylation with age. Young cultures were also hyper-methylated in gene promoters regulated by NF-κB and exhibited lower expression in genes that regulate the innate immune response, suggesting that methylation contributes to gene regulation in fibroblast innate response.

Previously, *TLR4* expression was shown to differ in the age-dependent fibroblast model, however, it was not known if variation in *TLR4* expression would affect mastitis severity. Therefore, fibroblasts were isolated from sixty lactating, adult Holstein cows and their expression of *TLR4*, along with LPS-induced production of IL-8 and IL-6, was used to rank the animals from high to low. Six high responders and six low responders were then experimentally infected in one mammary gland with *E. coli*. Overall, severity of mastitis was quite variable, with a few notable differences between high and low responders. High responding animals had an earlier increase in somatic cell count and febrile response that coincided with more efficient bacterial clearance. However, tissue damage and milk production did not differ between the two groups, indicating that while rapid up-regulation of the innate response addresses bacterial clearance, subsequent down-regulation is required to alleviate damage within the mammary gland.

Finally, one-week old bull calves were subjected to treatment with either saline or LPS to determine if neonatal exposure to endotoxin would make calves less responsive to a second LPS challenge at 32-days of age. The initial treatment showed a large effect of LPS as measured by higher plasma IL-6 and TNF-α concentrations in calves treated with LPS over saline. Subsequent treatment of all 10 calves with LPS showed a very similar response between the two treatment groups and significant inter-animal variability in clinical response. Fibroblasts and monocyte-derived-macrophages (MDMs) were also isolated following initial treatment to determine if any changes occurred at the cellular level as a result of LPS exposure. Fibroblasts isolated from calves at 20-days of age had a very low response to LPS that did not differ between the early life treatments. MDMs isolated from calves at 28-days of age were more responsive to LPS, but again no differences were detected between the early life treatments. In summary, our results suggest that DNA methylation likely plays a role in the cellular response to LPS and may partially contribute to differences between animals in severity of *E. coli* mastitis, however, the appropriate in vitro phenotype to detect susceptible animals still needs to be characterized before epigenetic biomarkers can be identified, and perhaps modified by environmental interventions.

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# CHAPTER 1: COMPREHENSIVE LITERATURE REVIEW

## 1.1 Bovine Mastitis: Prevalence and Economic Impacts

Mastitis, simply defined as inflammation of the mammary gland, is a significant source of economic losses in the dairy industry. It is a disease that shows worldwide prevalence as evidenced by numerous reports detailing the impacts of mastitis in every inhabitable continent (Verbeke et al., 2014, Boujenane et al., 2015, Oliveira et al., 2015, Yang et al., 2015). According to the 2014 USDA NAHMS Dairy Report, nearly a quarter of all cows (24.8%) experienced a clinical case of mastitis in the United States in 2013, ranging from 25.6%, 16.4% and 26.9% in small, medium and large operations, respectively (USDA, 2014). In terms of economic effects, a single case of clinical mastitis that occurs within the first 30 days of lactation costs the producer $444 (Rollin et al., 2015). In addition, throughout the entire 305-day lactation period, primiparous cows cost $325 per case of mastitis and multiparous cows cost $426 (Liang et al., 2017). The main source of economic losses is due to lowered milk quantity and non-saleable milk, but also include direct costs such as veterinary care, treatment, additional labor and early culling of high-producing animals (Liang et al., 2017). Furthermore, clinical mastitis has a deleterious effect on cow fertility, which reduces farm productivity and profitability, and it has been shown that the negative economic impacts of lowered fertility are especially high among cows infected with Gram-negative pathogens, *Escherichia coli* (*E. coli*) and *Klebsiella* spp. (Hertl et al., 2014, Fuenzalida et al., 2015).

## 1.2 Causative Agents of Mastitis

Mastitis is caused by a large variety of etiologic agents that are mainly bacterial in origin. To highlight the diversity of microorganisms that can cause mastitis, researchers in the United Kingdom analyzed the causative agent in 6,005 milk samples from clinical (43.8%) and subclinical (56.2%) mastitis cases and reported over 100 different organisms, with *E. coli, Streptococcus uberis* (*S. uberis*) and *Staphylococcus aureus* (*S. aureus*) being the three most common bacteria isolated (Payne, 2013). While *E. coli* and *S. aureus* are widely regarded as the two most common-mastitis causing pathogens, pathogen prevalence is also dependent on geographic location, management practices and identification method. A recent Finnish study based on PCR identification rather than culture-based methods revealed coagulase-negative Staphylococci (CNS) as the most prevalent pathogen among 93,529 cows with clinical or subclinical mastitis (Vakkamaki et al., 2017), while in the United States non-traditional laboratory culture showed greater prevalence of *Streptococcus* spp. and *E. coli* (Ganda et al., 2016). However, the latter study contradicts earlier data generated from >100,000 milk samples submitted to the Wisconsin Veterinary Diagnostic Laboratory, whereby CNS represented the largest proportion (13.22%) of mastitis-causing pathogens by standard culture-based methods (Makovec and Ruegg, 2003), a result that is more consistent with the known prevalence of CNS (Vanderhaeghen et al., 2014).

Mastitis-causing pathogens are also sub-divided into contagious and environmental pathogens. Contagious pathogens, such as *S. aureus*, are transmitted between cows, while environmental pathogens, such as *E. coli*, are acquired from the environment. Improvement of management practices in regards to herd hygiene through

mastitis control plans, such as the Five Point Plan developed in the 1960s, has alleviated much of the effect of contagious pathogens. However, opportunistic, environmental pathogens remain a significant and increasing concern for the dairy industry. Data from 741 clinical mastitis cases across 50 Wisconsin dairy farms showed that environmental species of bacteria (*E. coli*, *Klebsiella,* and environmental streptococci) represented 42.2% of all mastitis cases, with very few being caused by the contagious pathogen *S. aureus* (2.8%) (Oliveira et al., 2013). This data is representative of most large, modern dairy farms in that management practices have reduced cow-to-cow transmission of mastitis causing pathogens, however, the ability to deal with environmental pathogens remains a significant challenge.

### 1.3 Between-Animal Variation in Mastitis Severity

Mastitis severity is highly variable, and among one of the first observations describing the range in severity of mastitis was in the 1950s, when J.M Murphy (1956) noted that clinical stages of mastitis ranged from non-clinical to mild to severe. He also mentioned that while there were four major causative agents of mastitis, the severity of mastitis within the different organisms did not occur at the same frequency, whereby some organisms typically caused mild infection while others caused more severe infection. His observations remain mostly true today in that clinical mastitis can be mild, with only a few milk abnormalities, moderate, with changes in milk and local udder swelling, or severe, which occurs with systemic signs of disease. Infections can also be subclinical, resulting in no obvious signs of infection. As Murphy alluded to, severity of mastitis does not occur equally among causal organism. Subclinical infections are

typically caused by Gram-positive pathogens, such as coagulase-negative Staphylococci and *S. aureus* (Oliver et al., 2004, Thorberg et al., 2009). Conversely, environmental pathogens, such as *E. coli*, rarely persist and more frequently result in severe infection (Oliveira et al., 2013). However, even among bacterial pathogens that typically cause clinical signs, the severity of infection differs between animals and is shown to be heavily reliant on host variables, such as age, stage of lactation, and parity of the animal (Shuster et al., 1996, Burvenich et al., 2003, Burvenich et al., 2007). Additionally, there is evidence of a dose-dependency in the probability of infection, whereby different doses of bacteria cause variable rates of infection in experimental challenges (Bramley, 1976), indicating that a threshold of bacteria must be reached that supersedes innate mammary defenses to cause infection.

Bacterial strain type is another factor that has the potential to determine the severity of infection, however, reports showing whether this occurs in mastitis caused by *E. coli* have been equivocal. Recently, it has been suggested that experimental bovine and murine mastitis caused by environmental versus mammary pathogenic strains of *E. coli* results in mastitis that differs in severity as evidenced by elevated milk cytokine levels and somatic cell count in animals infected with pathogenic strains versus a commensal strain (Blum et al., 2017, Roussel et al., 2017). However, an assessment of 25 *E. coli* genomes, including 16 mammary associated strains, 8 fecal commensal strains and one milk commensal strain, showed well-known *E. coli* virulence factors were present in both mastitis-causing and fecal commensal strains. Furthermore, it was shown that genome content is significantly associated with the likelihood of virulence or fitness factors regardless of pathotype and that pathotypes are dispersed across phylogenetic groups.

The lack of an association between mammary associated strains and well-known virulence factors suggests that commensal and mastitis associated strains may be equally able to cause infection and likely cause infection through a variety of mechanisms, however, this hypothesis should be confirmed experimentally (Leimbach et al., 2017). In addition, even under experimental conditions, where cattle from different breeds were challenged with the same *E. coli* strain, severity of infection varied within and between breeds, indicating that the host response has a substantial role in infection outcome (Bannerman et al., 2008).

### 1.4 Immune Response to Mastitis

Knowledge of how the immune system responds to mammary gland infection is critical to understanding how between-animal variation in severity of mastitis is regulated. As previously mentioned, the magnitude of the immune response and the mediators that are activated are highly pathogen dependent. Transcriptional analysis comparing the very early innate response events that occur within the udder post infection with *E. coli* and *S. aureus*, the two-major mastitis-causing pathogens, has shown a clear difference in their magnitude of response. Just one hour after experimental infection, *E. coli* induces a response that is greater than 25 times stronger than *S. aureus* and also enhances expression of several immune response genes that are not activated by *S. aureus* (Petzl et al., 2016). This difference in early innate response is indicative of the progression of infection mediated by the two pathogens, whereby *E. coli* induces a rapid, acute, highly inflammatory response and *S. aureus* tends to elicit a more subdued response that can lead to chronic infection.

In a second, similar study, whole transcriptome analysis of *E. coli* and *S. aureus* infection (1-3 hrs. post-infection) showed the rapid induction of pro-inflammatory genes by *E. coli* that are regulated by LPS, TNF-α and IL-1ß signaling pathways (Gunther et al., 2017a). Again, this response is consistent with many reports that indicate *E. coli* induces the rapid activation of Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs) to activate pro-inflammatory gene transcription resulting in acute mastitis (Petzl et al., 2008, Schukken et al., 2011). Conversely, *S. aureus* failed to activate the majority of pro-inflammatory signaling pathways and instead the response was found to be regulated by immune-dampening transcription factors, TGFB1, ESR1 and SFR. Bacterial invasion and cytoskeletal rearrangements were also detected in *S. aureus* infected cell cultures, a result that was not seen in similar *E. coli* treated cultures (Gunther et al., 2017a). The ability for *S. aureus* to invade epithelial cells is likely dependent on its activation of immune-dampening mechanisms within the udder. Interestingly, *E. coli* gains the ability to invade epithelial cells in mice deficient in TLR4/LPS signaling (Elazar et al., 2010). This key difference in inflammatory response versus immune-dampening mechanisms explains the much higher prevalence of chronic *S. aureus* infections and acute *E. coli* infections.

Pathogen dependent differences aside, the early cellular responses to mastitis-causing pathogens are largely activated by sentinel cells that reside within the mammary gland, the majority of which are mammary epithelial cells and some resident leukocytes and lymphocytes. Epithelial cells are known to rapidly respond to infection by increasing expression of pro-inflammatory cytokines, cathelicidins, and acute phase proteins prior to leukocyte infiltration (Brenaut et al., 2014, Cubeddu et al., 2017). In addition, stromal

fibroblasts have recently been shown to be activated during mastitis infection and exhibit cross-talk with mammary epithelial cells (Chen et al., 2016, Zhang et al., 2016). Following activation of sentinel cells within the mammary gland, the main cellular responders are neutrophils, followed by monocyte-derived-macrophages and natural killer cells (Sipka et al., 2016). The infiltrating leukocytes are responsible for the increase in somatic cell count (SCC) that is quickly seen following a mastitis infection. Moreover, neutrophils are the primary leukocyte that are responsible for bacterial killing through the release of a large arsenal of antimicrobial proteases, peptides, reactive oxygen species, and neutrophil extracellular traps (Pechous, 2017). Ideally, bacterial killing by neutrophils will be short-lived and effective to clear bacteria but with their effects limited appropriately to reduce the cytotoxic effects of the neutrophil-derived molecules.

The adaptive immune response has been relatively understudied in comparison to the innate response in bovine mastitis. However, the production of the IL-17 family of cytokines (IL-17A, IL-17F and IL-22), typically produced in abundance by CD4 T helper cells of the Th17 lineage, although also produced by innate lymphoid cells called ILC3s, are up-regulated following intra-mammary challenge with *E. coli* and *S. aureus*, a finding that is in line with a predominantly neutrophilic response. In addition, it has been shown that IL-17 has synergistic effect on the production of a number of pro-inflammatory and chemotactic proteins in cellular and in vivo mastitis models (Roussel et al., 2015, Rainard et al., 2016) and aids in neutrophil-dependent bacterial clearance (Porcherie et al., 2016). Therefore, Th-17 cells are likely to contribute to the mammary gland response to mastitis. Knowledge of how other $CD4^+$ and $CD8^+$ T-cell subtypes respond are limited; however, it is known that $CD8^+$ cells proliferate and increase in their proportion in mammary

glands infected with *E. coli* (Mehrzad et al., 2008). Interestingly, in a recent study it was shown that antibodies seem to be somewhat inessential to mammary gland response to *E. coli* mastitis (Herry et al., 2017), a finding that is line with previous work where large differences in milk IgG response were measured in response to experimental *S. aureus* mastitis, with no difference in bacterial clearance (Benjamin et al., 2015b).

### 1.5 Inflammatory Response Regulation: What is the Appropriate Response?

The cellular and non-cellular components of immunity that define the appropriate response depend on the type of infecting organism (e.g. bacterial, viral or eukaryotic) and the location of intrusion. Response to infection is regulated at the level of gene transcription, mRNA stabilization and processing, protein translation, and through post-translational modifications, such as protein phosphorylation. Dysregulation of any of these steps may influence the effective response to infection, potentially resulting in more severe pathology or lowered ability to control infection. Under normal circumstances, bacteria are prevented from entering the mammary gland through physical barriers provided by the teat canal, including a keratin plug that seals off the mammary gland from the outside environment during the dry period and between milkings. Variation in cow and environmental factors appears to influence the probability that pathogens breach these physical barriers. For example, cow teat shape and teat sphincter length and patency are associated with pathogen specific susceptibility to infection, and reducing teat end bacterial contamination through environmental and udder hygiene practices reduces infection risk. If bacteria breach the physical barriers and escape killing by humoral milk

components, a prompt leukocyte response is required to neutralize bacteria before uncontrolled growth can occur (Rainard and Riollet, 2006).

The adequate response by the neutrophil has been the subject of years of discussion. In numerous reports it has been suggested that a rapid neutrophil response is essential to limiting severity of infection (Burvenich et al., 2003) and that during parturition, when an animal is very susceptible to severe mastitis, host neutrophil response is either suppressed or insufficient resulting in unnecessary damage to the mammary gland (Vangroenweghe et al., 2005b). Differences in the kinetics of neutrophil influx could be regulated at the level of extravasation. Successful neutrophil diapedesis depends on the interaction of L-selectin with selectin molecules on endothelial cells and by subsequent up-regulation and binding of $ß_2$-integrins (CD11a, b, c & CD18) with ICAM molecules on endothelial cells. An earlier report assessing the role of neutrophil CD11a and CD11b expression in cows that experience severe versus moderate responses to mastitis did not find a significant difference in receptor expression, however, they noted a negative correlation between the severity of mastitis and total circulating pre-infection neutrophil numbers and percent of both mature and phagocytic neutrophils in blood (Dosogne et al., 1997). A number of other studies have researched the role of $ß_2$-integrins and L-selectin molecules on neutrophil function during parturition or in response to *E. coli* or LPS-induced mastitis, with inconclusive results (Diez-Fraile et al., 2002). Neutrophil properties, such as pre-infection neutrophil viability (Mehrzad et al., 2004), elevated production of reactive oxygen species (Mehrzad et al., 2002) and increased expression of genes downstream of the TLR4 signaling (Stevens et al., 2011) have all been implicated in reducing severity of mastitis. In addition, both a causal and

protective role have been hypothesized for complement protein and neutrophil chemoattractant C5a. Expression of the C5a receptor (C5aR) is reduced in neutrophils from early lactation cows, however, high concentrations of C5a contribute to excessive cytokine production that is ultimately detrimental to the host and can result in sepsis-related complications typical of very severe mastitis (Stevens et al., 2012).

In contrast to previous reports of immune-suppression during the transition period, an overzealous innate immune response has also been associated with an increase in mastitis severity during parturition. Mononuclear cells isolated from early lactation cows produce significantly more TNF-α in response to LPS in comparison to mid-lactation cows (Sordillo et al., 1995). Induction of endotoxin tolerance within the mammary gland has been shown reduce the severity of experimental mastitis for at least 72 hours post LPS pre-treatment, reducing SCC influx and febrile response and increasing bacterial clearance. The protective effect of tolerance was shown to occur with the concurrent down-regulation of CXCL8, SAA3 and IL-6 (Petzl et al., 2012). Similarly, treatment with a steroidal anti-inflammatory together with antibiotic treatment reduces the extent of mastitis severity in comparison to no treatment and to antibiotic treatment alone (Sipka et al., 2013). Finally, animals ranked as high responders based on their fibroblast production of IL-8 exhibit a prolonged neutrophil response to experimental *E. coli* mastitis that delays their recovery to pre-infection milk production levels and increases tissue damage within the mammary gland (Kandasamy et al., 2011)

### 1.6 Cellular Models for in vivo Response

Due to the inherent variability of outbred species, such as dairy cattle, it may be necessary to study a large number of animals to glean biologically relevant and significant data. However, it is also often not feasible at the financial or ethical level to experimentally infect a large number of cows. To address these issues, cellular models have been developed to study how an animal might respond in vivo through in vitro characterization of their cells. Numerous cellular models have been developed to study mastitis and other infectious diseases, however, each has their inherent limitations and applicability. Monocyte-derived-macrophages have been successfully used to model response to bovine tuberculosis, whereby virulent strains of *Mycobacterium bovis* induce expression of numerous pro-inflammatory genes to a greater extent in macrophages isolated from resistant versus susceptible cattle (Alfonseca-Silva et al., 2016). This result makes sense for *M. bovis* infection because the bacteria primarily infect macrophages and often escape killing through immune-dampening mechanisms. On the other hand, using macrophages to model mastitis infection does not seem to accurately portray disease progression. Bovine-derived macrophages, which may be useful to measure broad non-specific responses, have been shown to lack the ability to distinguish between the pathogen-specific immune responses elicited by *E. coli* versus *S. aureus* (Gunther et al., 2016b) and therefore, may not be a sensitive enough model to measure the variable responses that occurs between-animals to the same pathogen.

The mammary epithelial cell is another potential cell model that has been used extensively to study the pathogenicity of mastitis-causing organisms (Pang et al., 2017), along with the differential host innate immune response to various pathogens and sub-

strains of the same pathogen (Gunther et al., 2016a, Gunther et al., 2016b). Interestingly, a novel use for the primary bovine mammary epithelial cell has very recently been developed to identify new expression biomarkers from cows that were described as high or low responders based on antibody production in response to *Clostridium difficile* immunization, in an effort to produce milk with high levels of anti-*C. difficile* IgA (Hillreiner et al., 2017). Epithelial cells isolated from high responders, in agreement with antibody production in vivo, expressed higher levels of numerous innate response genes, including *CXCL8*, *CXCL5*, *C3* and *SAA3*.

To avoid primary sampling of mammary epithelial cells, bovine mammary epithelial cell lines have also been used to study the immune response to mastitis causing pathogens (Roussel et al., 2015) and to study the effects of haplotypes in response to the same pathogen (Meade et al., 2012). However, mammary epithelial cell line response to different strains of *E. coli* did not accurately reflect the murine response to the identical pathogens, whereby the P4 strain of *E. coli* caused the most severe infection, with high levels of milk cytokines, but elicited a much more subdued cellular cytokine response in comparison to other strains (Roussel et al., 2017). Epithelial cell lines may be useful for studying the overall biological response to pathogens as they are less variable than primary cells, but for the same reason may not be a good tool for understanding between-animal or between-pathogen responses.

The primary fibroblast is another, more easily accessible, cell type that responds to TLR agonists and bacteria and are reflective of host response to infection (Gunther et al., 2016b). Primary fibroblasts have been used to study the immunological mechanisms that constitute response to infection in a number of host species, including human

response to Chikungunya virus (Selvamani et al., 2014), non-natural host response to herpes subtypes with varying degrees of virulence (Rogers et al., 2007) and in bovine response to LPS in relation to mastitis (Kandasamy et al., 2011) and systemic LPS response (Green et al., 2011a). Furthermore, primary dermal fibroblasts have been utilized to discern animal-to-animal variation in mastitis severity. Production of IL-8 following fibroblast stimulation with synthetic TLR agonists (LPS and PAM2CSK4) has been used as a marker to categorize animals as high or low responders and subsequent experimental mastitis has shown that high responders respond more severely to *E. coli* or *S. aureus* infection, respectively (Kandasamy et al., 2011, Benjamin et al., 2015b). Given this, the primary dermal fibroblast is suggested to be the preferable cell model for their ease of isolation, amenability to cryopreservation, responsiveness, and repeatability. Furthermore, it has been shown that mammary stromal fibroblasts are an active cell in response to mastitis, in that fibroblasts isolated from glands infected with mastitis versus healthy glands exhibit a number of inflammation related changes and have the capacity to decrease ß-casein secretion and up-regulate expression of *TNF-α* and *IL-8* in unstimulated epithelial cells (Chen et al., 2016). Alternatively, activated epithelial cells can induce an inflammatory response in fibroblasts isolated from healthy cattle (Zhang et al., 2016).

### 1.7 Genetics of the Immune Response to Mastitis

Ideally, the producer would like to be able to select animals that are inherently less susceptible to mastitis, as has been done for milk production traits. Doing so would reduce the costs associated with mastitis treatment and lowered milk production, and decrease the use of antibiotics in food-producing animals. However, this is not a simple

task. Resistance to mastitis is undoubtedly highly polygenic, and traits associated with lower risk for one pathogen may increase the risk for other pathogens or even other infectious diseases. The correct phenotype for mastitis resistance is also difficult to elucidate in that mastitis is caused by such a varied group of microorganisms. Unfortunately, unlike milk yield, genetic heritability of clinical mastitis has been measured to be quite low. Heritability estimates have been reported anywhere from 0.02 to 0.10 depending on the model used (Lund et al., 1999, Rehbein et al., 2013, Govignon-Gion et al., 2016). Heritability based on characteristics that are correlated with mastitis, such as somatic cell count or somatic cell score (SCS), has shown improved estimates for heritability. Using a recursive model, based on the closest test-day before and the closest test-day after clinical mastitis, one study showed that SCS has heritability of 0.12 and 0.16, respectively, between test day one and test day two (Rehbein et al., 2013). Splitting test measurements among different stages of lactation improves heritability, albeit slightly, to 0.17 (Jamrozik and Schaeffer, 2012). However, although correlation between mastitis and SCS is high, in practice this may only work for chronic or subclinical infections. Somatic cell measurements are typically only taken on a monthly basis and would likely miss many short-lived, acute infections, such as those caused by *E. coli*. Genetic differences in immune response genes, resulting in differential mastitis severity, is another potential method for genetic selection. Genome-wide association studies (GWAS) have been performed to try to identify specific gene regions that are associated with risk of clinical mastitis. One GWAS found a number of QTLs with immune-relevant genes across nine chromosomes, confirming the polygenic nature of mastitis resistance (Tiezzi et al., 2015). Again, since the authors did not distinguish between pathogen type,

this may also reflect that the importance of genes is variable in response to different organisms. Other GWAS have tried to identify SNPs that are associated with increases in SCS and clinical mastitis, and found QTLs on chromosomes 14 (Wang et al., 2015), 18 (Brand et al., 2011) and 20 (Kadri et al., 2015), associated with genes such as *LIFR* and *CARD6.* Unfortunately, the functional consequences of genetic variation have not been reported for most SNPs and QTLs.

Interestingly, one study that identified different haplotypes based on five SNPs in the *CXCR1* gene challenged three different haplotype combinations with *Streptococcus uberis* to determine the effect of haplotype on severity of mastitis. Severity based on SCC, bacterial counts, mammary and milk scores was significantly higher in one homozygous haplotype and significantly lower in one heterozygous haplotype (Siebert et al., 2017). Notably, CXCR1 is the receptor for IL-8, and genetic differences in the *IL8* gene have also been found whereby two distinct haplotypes have been identified, one of which is more responsive to LPS in vitro (Meade et al., 2012) and to intravenous LPS treatment (Stojkovic et al., 2016). Furthermore, in response to in vitro stimulation with a viral agonist, the opposite haplotype has the higher phenotype, suggesting that the two haplotypes may have evolved based on exposure to bacterial or viral antigens (Stojkovic et al., 2017).

### 1.8 Regulation of Immunity by Non-Genetic Mechanisms

There is substantial evidence that regulation of gene expression also occurs through heritable non-genetic, or epigenetic, mechanisms. Epigenetic regulation has been shown to occur through modification of histone proteins, regulation by miRNAs, and through DNA modifications, such as CpG methylation and hydroxy-methylation. There

are also varying degrees on what is known about how each of these modifications functions in generating changes in immunity. For instance, very few studies have identified a role for 5-hydroxymethylcytosine (5-hmC) in the regulation of the immune response. However, relatively recent reports have shown that 5-hmC may contribute to the regulation of T-cell development and activity (Ichiyama et al., 2015), along with increased risk of systemic lupus erythematosus (Zhao et al., 2016). There are elevated levels of 5-hmC in isolated CD4+ T-cells from patients with systemic lupus erythematosus that results in a concurrent increase in gene expression of 131 genes, a result that is in agreement with previous findings that 5-hmC up-regulates the expression of genes through DNA de-methylation. In addition, 5-hmC was shown to contribute to T-cell differentiation (specifically Th1 and Th17 cells) through the up-regulation of lineage specific transcription factors and cytokines, mediated by the Tet2 de-methylating enzyme (Ichiyama et al., 2015).

Micro-RNAs (miRNAs or miRs) regulate the expression of genes post-transcriptionally through binding and de-stabilization of mRNA. There is substantially more data to support the role of micro-RNAs in the immune response and in particular, the regulation of TLR signaling in response to immune stimulus or infection (Drury et al., 2017). Activation of TLR4 signaling in primary human monocytes increases the expression of miR-187, which subsequently down-regulates pro-inflammatory cytokine production in an IL-10 dependent manner. It was shown that miR-187 specifically targets *TNF-α* and *IκBζ* mRNA, resulting in reduced expression of *TNF-α, IL-6* and *IL12-p40* (Rossato et al., 2012). Similarly, miR-146a inhibits TLR4 signaling through its interactions with *TRAF6* and *IRAK1*, and miR-146a is temporally regulated with miR-

155, another miRNA that enhances TLR4 signaling through its interactions with transcripts of inhibitory proteins *SHIP1* and *SOCS1* (Mann et al., 2017). Interestingly, expression of miR-146a is sufficient to induce endotoxin tolerance in the monocyte cell line THP-1 and it can elicit its effects in both the presence and absence of LPS (Nahid et al., 2009).

Numerous histone modifications have been identified that also contribute to the regulation of the innate and adaptive immune response. Histone acetylation, typically associated with transcriptional activation, contributes to cellular response to TLR3 ligands. Specifically, H3K9 and H4K8 acetylation is increased in cells following treatment with a TLR3 agonist poly (I:C) and is hypothesized to contribute to early HIV-1 trans-activation that occurs to a higher degree with TLR3 stimulation (Bhargavan et al., 2016). In addition, bronchial epithelial cells that are primed with IFN-γ exhibit increased expression of the antiviral protein RIG-I through the reduction of H3K9 tri-methylation (H3K9me3), a histone modification that represses gene expression. This epigenetic effect of IFN- γ on bronchial epithelial cells may partially protect against early-life susceptibility to viral infection and/or increased asthma-associated viral infections (Spalluto et al., 2017).

Finally, and by far the most extensively studied, is the role of DNA methylation in differential susceptibility and severity of infection and other inflammation-related disorders. In fact, TLRs themselves are frequently regulated by differential methylation, making them the ideal candidates for assessing mechanisms of immune response regulation (Hennessy and McKernan, 2016). It has been shown that infection with *Mycobacterium tuberculosis* induces extensive re-programming of methylation patterns

found within the *TLR2* promoter that results in changes in *TLR2* expression and also correlates with the extent and severity of infection (Chen et al., 2014). Chondrocytes isolated from patients with osteoarthritis versus healthy patient controls express significantly higher *IL-8* that occurs with decreased methylation of the *IL8* promoter and reduced transcription factor trans-activation (Takahashi et al., 2015). In summary, epigenetic modifications significantly impact how the immune system responds to infection. Epigenetic changes may also occur as the result of infection inducing an innate "memory" response, as is the case in endotoxin tolerance and "trained" immunity (Netea et al., 2016).

### 1.9 Epigenetic Regulation of Immunity in Agricultural Species

Epigenetic regulation of gene expression in agricultural species has been gaining attention in recent years and has led to advances in both our understanding of the biological consequences of epigenetic regulation and in the sequencing technologies needed to identify epigenetic modifications in animals of agricultural importance. One study has recently addressed the role of epigenetic regulation in bovine response to *Mycobacterium bovis*. In comparison to their healthy counterparts, animals infected with *M. bovis* acquire a number of DNA methylation changes within their $CD4^+$ T-cell population that is unique to their infected status. T-cells in infected animals become significantly hypo-methylated in a number of genes that are regulated by TGF-ß, a cytokine that regulates T-cell development and differentiation. In addition, the canonical $TH_1$ cytokine, *IFNG,* encoding IFN-γ, was discovered to be significantly hypo-methylated and up-regulated in infected animals, whereas, *TNFRSF4*, a gene that

transcribes the OX-40 co-receptor responsible for T-cell survival and proliferation, was hyper-methylated and significantly repressed (Doherty et al., 2016).

Treatment of bovine peripheral blood mononuclear cells (PBMCs) with LPS reduces the expression of key enzymes that mediate DNA methylation and histone de-acetylation, consistent with up-regulation of gene expression (Doherty et al., 2013). However, it is interesting to note that in the same study, treatment of PBMCs with trichostatin A (TSA), an inhibitor of the histone de-acetylase enzyme, decreased the expression of pro-inflammatory cytokines in response to LPS compared to untreated cells stimulated with LPS. Since TSA is a pan-HDAC inhibitor it is possible that up-regulation of anti-inflammatory proteins occurred as a result of treatment and over-shadowed the effects of LPS. In addition, the mixed cell population may have confounded the study, making these results difficult to interpret. It is thought that endotoxin tolerance is the result of epigenetic modifications, however, treatment of primary bovine epithelial cells with a number histone-modifying enzyme inhibitors also has very little effect on subsequent response to *E. coli* (Gunther et al., 2017b). These results suggest that histone modifications may play a lesser role than originally thought in response to LPS.

A number of epigenetic changes occur in the livers of animals challenged with *E. coli* and as a result of increased concentrations of LPS induced by sub-acute ruminal acidosis (SARA). Following *E. coli* mastitis, liver biopsies showed chromatin de-condensation in genes transcribing *TLR2*, *TLR4*, haptoglobin (*HP*) and lipopolysaccharide binding protein (*LBP*) along with increased expression. Interestingly, correlation of *TLR4* expression and chromatin remodeling was not as strong as for the other three genes, however, *TLR4* was also significantly hypo-methylated (Chang et al.,

2015a). Differential methylation of *TLR4* has also been shown to occur during SARA, a metabolic condition in ruminants that decreases rumen pH such that microbial death occurs and leads to the release of LPS into the circulation. When induced experimentally in goats and dairy cows, the systemic release of LPS leads to an enhanced expression of *TLR4* and cytokine response in the liver that occurs concurrently with *TLR4* chromatin de-condensation and a substantial decrease in promoter methylation (Chang et al., 2015c). In cows, increased hepatic expression of *HP*, *SAA3* and *LBP* also occurs with a significant reduction in promoter methylation and chromatin loosening (Chang et al., 2015b).

Differential methylation of *TLR4*, therefore, seems like a potential mechanism of gene regulation that may determine the susceptibility and severity of *E. coli* mastitis. Using the primary dermal fibroblast as a model cell type, it has been previously shown that as animals increase in age from 5- to 16-months, their fibroblasts exhibit a much higher response to in vitro LPS treatment and increased expression of *TLR4*. Age-dependent differences in fibroblast IL-8 production in response to LPS is also abolished upon treatment with the DNA methylation inhibitor 5-aza-2′deoxycytidine (AZA), indicating that DNA methylation plays a role in the age-dependent difference in response (Green et al., 2015). As these cells were isolated from the same animals at two separate ages and will not be confounded by genetic factors, this model is ideal for discovering epigenetic differences that regulate response to LPS.

### 1.10 Environmental Effects on Epigenetic Modifications and Immunity

In addition to the identification of genetic and epigenetic markers that confer susceptibility or resistance to severe mastitis, it is of interest to determine if epigenetic

differences can be altered through early life environmental interventions. If financially feasible, this would provide producers with another tool to lower the susceptibility of their animals to severe mastitis. There is considerable data to suggest that environmental exposures, particularly when they occur early in life, can influence disease susceptibility. For example, there is a substantial difference in the prevalence of asthma in Hutterite versus Amish children, whereby Amish children have a much lower risk of developing asthma (Stein et al., 2016). The lowered prevalence and increased protection against developing asthma is thought to occur as a result of a much higher (6.5-times) level of endotoxin measured in house-dust collected from Amish households, resulting in a more tolerant, hypo-responsive phenotype to everyday allergens. In support of this hypothesis, stimulation of peripheral blood leukocytes isolated from Amish and Hutterite children with LPS results in significantly lower cytokine concentrations in challenge supernatant from the Amish population. Furthermore, treating mice with house-dust collected from either Amish or Hutterite households showed a significant protective effect induced by Amish house-dust to airway hyperresponsiveness and eosinophilia and suppression of IgE and cytokine response to challenge with ovalbumin.

The aforementioned study is further supported by a number of other reports that have found an immune-tolerizing effect of early life endotoxin on adult immunity. Rat pups that were exposed to a single dose of LPS in early gestation exhibited lower leukocyte recruitment into their lungs following ovalbumin-induced experimental asthma (Kirsten et al., 2011). In addition, an earlier study showed that neonatal LPS treatment of rat pups at 14-days of age has a significant effect on a subsequent LPS exposure in adulthood. In comparison to their saline treated counterparts, LPS-treated pups had a

lower response to a second LPS exposure that was reflected in an attenuated circulating cytokine response along with a lower fever response. The authors attributed the LPS-induced suppression to increased corticosterone signaling in rats treated with LPS as pups (Ellis et al., 2005). Interestingly, although similar results were measured in LPS-induced circulating cytokine response in mice that were exposed to LPS at conception versus saline treated mice, no differences were measured in corticosterone levels, indicating that this may not be the only factor in determining neonatal LPS-induced suppression of adult innate immunity (Williams et al., 2011). Finally, calves treated with LPS in late gestation also exhibited changes to their innate immune response as young adults when exposed to a second treatment with LPS. LPS-treated heifers tended to be less effected by LPS, based on temperature response and sickness score, and also express higher levels of neutrophil ß-defensin genes (Carroll et al., 2017).

Although substantial evidence for the effect of early life endotoxin exists, few studies have addressed the mechanisms behind the phenotypic differences. One study, however, revealed a large change in the regulatory T-cell ($T_{reg}$) population found in cord blood of pregnant mothers exposed to a farm environment in comparison to unexposed mothers. The $T_{reg}$ population from farm-exposed mothers was more abundant and had an increased capacity to down-regulate effector T-cell function (Schaub et al., 2009). Notably, the change in $T_{reg}$ phenotype occurred in conjunction with decreased methylation and increased expression of *FOXP3*, a transcription factor that is necessary for $T_{reg}$ development.

Differences in early life maternal care have also been shown to affect epigenetic programming in adult animals. Rat pups reared by low maternal care dams, as measured

by lower levels of licking and grooming and arched back nursing, exhibit increased methylation and reduced expression of hippocampal glucocorticoid receptor that is reversible through cross-fostering or treatment with TSA, both of which result in de-methylation of the glucocorticoid receptor promoter region. The effect of maternal care on methylation of the glucocorticoid receptor was specifically attributed to differences in care in the first 6 days of life, and as a result, low maternal care adult rats exhibit a significantly higher corticosterone response to stress (Weaver et al., 2004). Interestingly, a similar difference in maternal care exists in the upbringing of Angus versus Holstein calves. In early life, Angus calves are allowed to remain with their dams and receive maternal care through nursing and grooming, whereas Holstein calves are separated from their dams soon after birth and raised by a calf handler in individual hutches. While not yet directly correlated to differences in maternal care, fibroblasts isolated from the two breeds exhibit disparate responses to LPS, with Holstein cows being much more hyper-responsive to the effects of LPS treatment (Benjamin et al., 2016a). How this difference is regulated, either epigenetically or otherwise, remains to be determined.

### 1.11 Unanswered Questions

The relationship between genetic, epigenetic, and environmental factors and their regulation of immunity in the bovine, especially how each of these contributes to mastitis severity, has yet to be fully elucidated. It is known that there is substantial inter-animal variation in mastitis severity to the same pathogen, such that some animals are quick to recover and others suffer from severe, systemic responses that may result in death of the animal. Genetic differences have been suggested to partially regulate the difference in mastitis susceptibility and severity, but for the most part have not been

tested in experimental models. The one exception to this is the role of *CXCR1* haplotypes in response to *S. uberis,* which may prove useful in future selection strategies (Siebert et al., 2017). However, it is very likely that susceptibility to severe mastitis is polygenic and pathogen-dependent. Clinical mastitis also has very low estimates of heritability, however, the majority of studies classify the incidence of clinical mastitis categorically, without addressing the severity of each case. As such, heritability of severe clinical mastitis may be higher than initially predicted.

It has also become increasingly clear that the optimum innate response is under-characterized and is not all or nothing. Many studies have used the transition cow as a model for a "severe responder", due to the increased risk of animals developing severe mastitis at parturition. These studies suggest that the transition cow is immune-compromised. Therefore, much past and on-going research on mastitis control focuses on boosting the immune system. However, mastitis severity is also associated with increased production of TNF-α, IL1-ß and IL-8 (Wenz et al., 2010), and studies suggesting overall immune-suppression fail to address the variability between animals in mastitis severity even during early lactation. Therefore, it is of the author's opinion that a dysregulated rather than immune-suppressed response be attributed to the increased risk of severe mastitis in transition cows. Efficient neutrophil response is necessary but it should not be so exuberant to cause excessive tissue damage. Therefore, identifying the factors that contribute to effective response and then recovery following infection are required, and this may be facilitated by access to an accurate cellular model.

Beyond genetics, the study of the phenotypic effects of epigenetic differences is still in its infancy in the bovine. As previously discussed, a variety of epigenetic

modifications have been shown to contribute to regulation of the immune response in several different species; as such, it is unlikely that this will be any different for the bovine and suggests there is significant room for improving genetic resistance to mastitis. Finally, it is of interest to identify treatments, such as dietary interventions or immune stimulants, that are easy to implement and can be utilized early in calf development to generate an adult phenotype that has a lower susceptibility to severe mastitis. In this case, training the immune response to effectively respond to a pathogen without creating excess damage or undermining immunity elsewhere, is highly desired.

To discover how genetic, epigenetic and the environmental factors collectively regulate the bovine innate immune response and potentially alter susceptibility to severe mastitis, I used a primary dermal fibroblast cell model to assess phenotypic differences between animals in their innate response to LPS. The dermal fibroblast is a readily accessible and TLR-responsive cell type that is useful for measuring the responses of a large cohort of animals. Furthermore, the cell model was used in conjunction with in vivo experiments to address my hypothesis:

> Between-animal variation in the severity of *E. coli* mastitis is partially mediated by differential methylation of genes that regulate the innate immune response, resulting in susceptible and resistant phenotypes that can be altered through early-life environmental interventions.

To first determine how DNA methylation regulates innate immunity, fibroblasts that exhibit an age-dependent up-regulation in their response to LPS were used to identify genome-wide differences in DNA methylation. As these cells were isolated from the same animals at 5- and 16-months of age and show clear differences in their response to

LPS, they are an ideal model to identify epigenetic differences that regulate the innate response without the confounding effect of genetic variables. Next, to identify how differential regulation of the TLR4 pathway contributes to mastitis severity, either at the genetic or epigenetic level, expression of *TLR4* and downstream signaling components were measured in fibroblasts isolated from a large cohort of lactating adult cows. Cows identified as either high or low responders were subsequently challenged with *E. coli* in an experimental mastitis model to determine if high responders would experience more severe mastitis, as was predicted.

Finally, since early life LPS exposure has been shown in numerous reports to down-regulate immunity, presumably through epigenetic mechanisms, neonatal bull calves were treated with either LPS or saline at one-week of age. Subsequent in vivo and cellular responses were then analyzed to determine the effects of early life treatment. If found to be beneficial, it may be of interest to producers to stimulate heifer calves at a young age with a low-dose of endotoxin, or another immune stimulant, to modulate heifer and adult cow response to *E. coli*. These works contribute to our understanding of epigenetic regulation of bovine innate immunity, addresses the utility of the fibroblast model to determine genetic and epigenetic biomarkers of mastitis resistance and, adds to our knowledge of neonatal exposures and their ability to modify adult phenotype.

# CHAPTER 2: GENOME-WIDE METHYLATION ANALYSIS REVEALS DIFFERENTIALLY METHYLATED LOCI THAT ARE ASSOCIATED WITH AN AGE-DEPENDENT INCREASE IN BOVINE FIBROBLAST RESPONSE TO LPS

## 2.1. Abstract

Background: Differences in DNA methylation are known to contribute to the development of immune-related disorders in humans, but relatively little is known about how methylation regulates immune function in cattle. Utilizing whole-transcriptome analyses of bovine dermal fibroblasts, we have previously identified an age and breed-dependent up-regulation of genes within the toll-like receptor 4 (TLR4) pathway that correlates with enhanced fibroblast production of IL-8 in response to lipopolysaccharide (LPS). Age-dependent differences in IL-8 production are abolished by treatment with 5-aza-2-deoxycytidine and Trichostatin A (AZA-TSA), suggesting epigenetic regulation of the innate response to LPS. In the current study, we performed reduced representation bisulfite sequencing (RRBS) on fibroblast cultures isolated from the same animals at 5- and 16-months of age to identify genes that exhibit variable methylation with age. To validate the role of methylation in gene expression, six innate immune response genes

that were hyper-methylated in young animals were assessed by RT-qPCR in fibroblasts from animals at different ages and from different breeds.

Results: We identified 14,094 differentially methylated CpGs (DMCs) that differed between fibroblast cultures at 5- versus 16-months of age. Of the 5065 DMCs that fell within gene regions, 1117 were located within promoters, 1057 were within gene exons, 2891 were within gene introns, and 67% were more methylated in young cultures. Transcription factor enrichment of the promoter regions hyper-methylated in young cultures revealed significant regulation by the key pro-inflammatory transcriptional enhancer, NF-κB. Additionally, five out of six chosen genes (*PIK3R1, FES, NFATC1, TNFSF13,* and *RORA*) that were more methylated in young cultures showed a significant reduction in expression post-LPS treatment in comparison with older cultures. Two of these genes, *FES* and *NFATC1,* were similarly down-regulated in Angus cultures that also exhibit a low LPS response phenotype.

Conclusions: Our study has identified immune-related loci regulated by DNA methylation in cattle that may contribute to differential cellular response to LPS, two of which exhibit an identical expression profile in both low-responding age and breed phenotypes. Methylation biomarkers of differential immunity may prove useful in developing selection strategies for replacement cows that are less susceptible to severe infections, such as coliform mastitis.

Keywords: DNA Methylation, Innate Immunity, LPS, Inflammation, RRBS

## 2.2. Introduction

The innate immune response is an organism's first line of defense against pathogenic microorganisms and the primary response to tissue damage. Innate immunity

is mediated by specialized leukocytes and other cells that serve as a barrier to the environment, such as epithelial cells and dermal fibroblasts. Innate immune cells are characterized by their expression of germ-line encoded pattern recognition receptors (PRRs), which recognize conserved molecular patterns (PAMPs), such as lipopolysaccharide found on the outside of Gram-negative bacteria and viral nucleic acids, and elicit the appropriate defense response upon recognition. The most thoroughly characterized of these PRRs are Toll-like receptors (TLRs), a family of type-I transmembrane proteins first discovered in *Drosophila melanogaster* when the *Toll* gene was found to be essential for bacterial and fungal defenses (Lemaitre et al., 1996, Michel et al., 2001). Upon TLR-ligand binding, an intracellular signaling cascade is activated that allows for the transcription of numerous pro-inflammatory proteins, such as IL-6, TNF-α and IL1-β, through the action of transcription factors NF-κB, AP-1, and Interferon Response Factors (IRFs), among others (De Nardo, 2015). While it is clear that the response elicited by TLRs and other PRRs are crucial to the defense against a variety of bacterial, viral and eukaryotic pathogens (Sturdevant and Caldwell, 2014, Sacramento et al., 2015, Uchiyama et al., 2015), an exacerbated or dysregulated innate response also contributes to infection related inflammatory disorders, such as in antibody dependent enhancement and the subsequent "cytokine storm" characteristic of Dengue hemorrhagic fever (Callaway et al., 2015). In addition, the type I interferon response, normally beneficial to the host during a viral infection, has been shown to actually increase susceptibility to some bacterial, viral and protozoan infections (Stifter and Feng, 2015). The innate immune response is also a key contributor to a number of sterile pro-

inflammatory conditions, such as rheumatoid arthritis and asthma (Starkhammar et al., 2015, Lacerte et al., 2016).

The severity of bovine mastitis, a primary cause of economic losses to dairy milk production, is similarly more often associated with increased production of pro-inflammatory mediators, such as, TNF-α, IL1-ß, and IL-6, than it is with the number of bacteria present within the mammary gland. Mastitis severity has also been shown to vary greatly, even under controlled experimental settings in which pathogen factors are held constant. This suggests host factors play a significant role in mastitis outcome (Kornalijnslijper et al., 2003). Several studies have shown a dysregulated population of milk and serum neutrophils, along with greater concentrations of TNF-α and reactive oxygen species, during the peri-partum period when animals are highly susceptible to severe mastitis (Ballou, 2012). Interestingly, priming of the mammary gland with 1 µg of LPS, which causes a substantial but short-lived inflammation, reduces severity of experimentally induced *Escherichia coli* (*E. coli)* mastitis 10 days post LPS priming (Petzl et al., 2012). In an effort to explain the mechanism behind this reduction in mastitis severity, a study by Gunther et al. (Gunther et al., 2012) measured gene expression in response to heat killed *E. coli* in primary mammary epithelial cells following pre-treatment with LPS. Priming of cells was found to increase expression of ß-defensins while decreasing pro-inflammatory and apoptotic gene expression, including *IL1-ß*, *TRAF6*, and TNF superfamily proteins (*TNFSF13B* and *TNFSF10*), indicating that the previously measured benefit of LPS priming in an intra-mammary infection with *E. coli* was due in part to a reduction in inflammatory response (Gunther et al., 2012). In a murine mastitis model, administration of LPS into the mammary gland of *TLR4*(-/-) mice

to induce mastitis showed that while knockout mice had significantly lower serum cytokines CXCL1, IL1-ß, IL-6, IL-10, TNF-α, CCL2 and IP10, they also experienced a lower degree of mammary gland involution while maintaining increased capacity for milk production compared to wild-type littermates (Glynn et al., 2014). In agreement with the aforementioned studies, our lab has shown that high responding dairy animals, based on dermal fibroblast IL-8 production in response to TLR ligands, have greater tissue damage and neutrophil influx into the mammary gland post-infection and a slower return to pre-infection milk production levels in response to both *Staphylococcus aureus* (*S. aureus*) and *E. coli* mastitis, without any benefit to their ability to clear bacteria (Kandasamy et al., 2011, Benjamin et al., 2015b).

Several factors, including genetic differences, may contribute to variation within an animal's infection response phenotype. In one example, single nucleotide polymorphisms in the *TLR4* gene have been linked to increased susceptibility to *Mycobacterium avium subspecies tuberculosis*, or Johne's disease, in Canadian Holsteins (Sharma et al., 2015). However, a recent study between 3 breeds indicated an estimated heritability of clinical mastitis to be between 2% and 4%, suggesting a very large role for non-genetic factors (Govignon-Gion et al., 2015). Epigenetic modifications, such as DNA methylation, may help to explain discrepancies measured between large phenotypic differences with little genetic basis. In agreement with this, DNA methylation in two breeds of chicken has been shown to contribute to differential susceptibility to infectious diseases such as avian flu and *Salmonella* infection. Whole genome bisulfite sequencing (WGBS) revealed greater than 5,000 differentially methylated regions (DMRs) in lung tissue between the two breeds, with a portion of DMRs falling within gene regions. The

methylation data generated by WGBS together with mRNA expression data generated by RNA-seq, also identified genes with both differential methylation and expression, most notably *TLR4* and *PIK3CD* (Li et al., 2015).

In support of epigenetic regulation of the bovine innate immune response, data generated in our lab has shown that an age-dependent increase in dermal fibroblast response to LPS is ameliorated following treatment with a DNA methylation and histone deacetylase inhibitor, AZA-TSA (Green and Kerr, 2014). A subsequent study was performed to identify methylation and gene expression differences in fibroblast cultures isolated from female calves at 5- and 16-months of age. Transcriptome-wide analysis of these two sets of cultures identified numerous genes within the TLR4 response pathway that were up-regulated in older, more responsive cultures, including *TLR4, CD14, IL-8* and *TNF-α* (Green et al., 2015). In the same study, we sought to identify changes in DNA methylation between the young and old cultures that contribute to age-related differences in innate immunity, however, the MIRA (methylated CpG island recovery assay)-seq technique that was utilized does not have the resolution required to determine methylation differences at the base pair level. As such, very little difference in methylation was found between the two sets of cultures.

Another phenotypic difference we have identified is the differential response between Holstein and Angus breeds, in which Holstein cattle exhibit a much higher fibroblast response to LPS than Angus cattle. The few reports of mastitis in beef cows have also suggested beef breeds are less susceptible to coliform mastitis, with no cases of *E. coli* mastitis cases detected in several studies (Lents et al., 2002, Persson Waller et al., 2014). In this case, genetic differences between breeds are likely to have an influence.

However, calves from these two breeds experience highly disparate upbringing. In contrast with Angus and other beef breeds, Holstein calves are immediately separated from their dam, allowing for little to no maternal care, which has been shown to contribute to differential methylation of hormone receptors in rats (Pena et al., 2013). Again, transcriptome analysis of age-matched fibroblasts isolated from the two breeds revealed a number of pro-inflammatory genes with differential expression, with Holstein cultures exhibiting significant up-regulation as compared to Angus cultures. However, little to no difference in DNA methylation was found using the MIRA-seq technique (Benjamin et al., 2016a).

Knowledge of epigenetic regulation of the immune response in dairy cattle is currently lacking, and will be important to understanding differences in mastitis susceptibility. From the previous studies, it is clear that methylation differences at a base pair resolution are required. As such, the aim of the current study was to identify base-pair specific DNA methylation differences in fibroblasts taken from Holstein heifers at 5- and 16-months of age, using reduced representation bisulfite sequencing (RRBS). Once candidate differentially methylated genes were identified, we determined whether methylation affects gene expression by measuring fibroblast gene expression at various time points post-LPS treatment. Furthermore, we determined whether genes regulated by methylation in young and old cultures also differed in gene expression between Angus and Holstein cultures. Overall, the current study contributes to our knowledge of candidate immune response genes that are subject to regulation by DNA methylation within the genome of dairy cattle and may lead to a better understanding of the mechanisms that regulate inter-animal variation in susceptibility to severe mastitis.

## 2.3. Materials and Methods

### 2.3.1 Experimental Animals

Six pairs of dermal fibroblast samples, collected from Holstein heifers at 5 and 16 months of age, were used from a previously characterized cohort of 15 heifer fibroblast samples (Green et al., 2011a). Primary dermal fibroblast collection and ranking based on IL-8 production following *in vitro* LPS stimulation was previously described (Green et al., 2011a). The six pairs of fibroblast samples chosen for DNA methylation analysis were all selected from mid-responding animals to reduce inter-animal variation within the two groups that could potentially interfere with any true age effects.

A second set of dermal fibroblasts was collected for the breed difference experiments. Fibroblasts were isolated from four 19-month Holstein and four 19-month Black Angus cows. Fibroblast collection, isolation and LPS response are described elsewhere (Benjamin et al., 2016a).

In both the age and breed experiments, fibroblasts were stimulated with 100 ng/ml of LPS and RNA isolation was performed side-by-side in neighboring wells. From the same vial of cryopreserved cells used in the LPS challenge, some cells were cultured in a T-75 $cm^2$ culture flask for DNA isolation. Cells were again cultured side-by-side and untreated cultures were used for DNA extraction.

All experiments were approved by the Institutional Animal Care and Use Committee at the University of Vermont.

### 2.3.2 Fibroblast Culturing and LPS Challenge

Growth media for fibroblast cultures was Dulbecco's Modified Eagle Media (DMEM; Hyclone Laboratories, Logan, UT) supplemented with 5% fetal bovine serum

(FBS; Hyclone Laboratories), 1% penicillin-streptomycin (Hyclone Laboratories) and 1% insulin-transferrin-selenium (ITS; Mediatech Inc., Herndon, VA). Fibroblasts were revived from cryopreservation and expanded in a T-75 $cm^2$ culture flask at 37°C 5% $CO_2$ in a humidified incubator. Confluent flasks were then treated with 0.25% trypsin (MP Biomedical, Santa Ana, CA) and cells were transferred to a 6-well plate at 1.25 x $10^5$ cells/ml in a total volume of 2 ml, or 2.5 x $10^5$ total cells and cultured for 24 hours for the LPS challenge experiments. Remaining cells were transferred to a T-75 $cm^2$ culture flask and allowed to grow to confluency for subsequent DNA isolation.

After 24 hours, cells in the 6-well plates were treated with 100 ng/ml of ultra-pure LPS isolated from *Escherichia coli* O111:B4 (Sigma-Aldrich, St. Louis, MO) for either 0, 2, 8 or 36 hours. At 0 and 36 hours, media was collected for protein production analysis. After removal from the wells, media was centrifuged at 10,000 *x* g for 1 minute to remove cellular debris and stored at -20°C until further analysis. At every time point, cells were rinsed gently with Dulbecco's Phosphate Buffered Saline (DPBS; Hyclone Laboratories) and cell lysate was collected by adding 500 µl cell lysis buffer (5 Prime, Hamburg, Germany) to the well. Cell lysate was stored at -20°C until RNA isolation. Cells that were grown in a T-75 $cm^2$ culture flask for DNA isolation were treated with trypsin at confluency and centrifuged at 400 *x* g. The cell pellet was then lysed with 300 µl cell lysis buffer (5 Prime) by adding the lysis buffer and vortexing for 15 seconds. The cell lysate was stored at -20°C until DNA isolation.

### 2.3.3 Quantification of IL-8 and IL-6 Protein

Interleukin-8 production in media from LPS stimulated dermal fibroblasts was determined with a sandwich ELISA (Mabtech Inc., Cincinnati, OH) per manufacturer's

protocol with slight modifications. Capture antibody was diluted 1:500 to 1 µg/ml in 0.05 *M* bi-carbonate buffer. Recombinant bovine IL-8 (Thermo Scientific, Rockford, IL) was used as the assay standard with a detection limit of 156.25 pg/ml. Capture antibody was diluted 1:20,000 in PBS-0.05% Tween-20 to a concentration of 0.025 µg/ml. Streptavidin-horseradish peroxidase was diluted 1:15,000 in PBS-0.05% Tween-20 to a concentration of 0.07 µg/ml.

Interleukin-6 production was also determined by a sandwich ELISA (Thermo Scientific) per manufacturer's instructions. Briefly, capture antibody was diluted 1:100 in 0.05 *M* bi-carbonate buffer. Recombinant bovine IL-6 (Thermo Scientific) was used as the assay standard with a detection limit of 156.25 pg/ml. Detection antibody was diluted 1:100 in PBS-0.1% BSA. Streptavidin-horseradish peroxidase (Sigma-Aldrich) was diluted 1:2000 in PBS-0.1% BSA to a concentration of 0.5 µg/ml. Development of IL-8 and IL-6 ELISA plates was done by adding 3,3',5,5'-tetramethylbenzidine substrate (TMB; Thermo Scientific) to the wells and the reaction stopped with 1 *M* $H_2SO_4$.

### 2.3.4 DNA extraction and preparation for RRBS

DNA was extracted from 5- and 16- month dermal fibroblasts using a 5-Prime PerfectPure™ Archive DNA Extraction Kit (5 Prime) per manufacturer's protocol. DNA concentrations were then determined using a Qubit™ 2.0 Spectrofluorometer (Life Technologies, Carlsbad, CA). DNA was diluted to 40 ng/µl and a total of 2 µg was sent to Zymo Research (Irvine, CA) for Methyl-MiniSeq™ RRBS library preparation. Libraries were generated with 200-500 ng of DNA as previously described (Kanzleiter et al., 2015). Briefly, DNA was sequentially digested with 60 units of TaqαI and 30 units of *MSP*I (NEB, Ipswich, MA), which recognizes CCGG as a cut site and cleaves after the

first cytosine, creating DNA products with CG dinucleotides on both ends of the DNA and enriching for CG rich regions. Following enzymatic digestion, DNA products were end-repaired, A-tailed, and extracted with DNA Clean and Concentrator Kit™ (Zymo Research). The extracted DNA was then ligated to methylated Illumina primers using the Illumina DNA preparation kit and protocol (Illumina, San Diego, CA). Adaptor ligated DNA was then size selected for desired input fragments (150-250 bp and 250-350 bp) with a 2.5% NuSieve 3:1 agarose gel and extracted using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research). Successfully ligated and purified DNA was then bisulfite converted using the EZ DNA Methylation-Lightning™ Kit (Zymo Research). Control DNA was similarly bisulfite converted to assess conversion rates, which were 99% for all samples. Following conversion, preparative-scale PCR was performed with a total of 16 cycles and PCR products were purified with the DNA Clean and Concentrator Kit™ (Zymo Research). Individual DNA libraries were then sequenced on the Illumina HiSeq2000 (Illumina), generating 50 bp paired-end reads and base-calling was performed using standard Illumina base-calling software.

### 2.3.5 DNA Sequence Processing and Alignment

Following sequencing, bioinformatics analysis was performed at Zymo Research using a proprietary analysis pipeline written in Python. Prior to alignment, reads were assessed for quality using FastQC (v0.11.1, Babraham Bioinformatics, UK) and Trim Galore (v0.3.7, Babraham Bioinformatics, UK). Bases with a Phred score greater than 20 were kept for downstream analysis (*--paired --phred33 -q 20)*. Illumina adaptor trimming was done using Trim Galore with the default settings which are automatically set to standard Illumina adaptors unless otherwise specified. Trim Galore also contains a

setting specifically for trimming RRBS data (*--rrbs*), which was used to further modify our reads. RRBS introduces artificial CpG sites which require trimming in order to avoid them being used in methylation calling. To do so, Trim Galore trims the first 2 bases from the 3' end of the sequence so the C closest to the second enzyme cut site is not included in methylation calling. Reads were then mapped to an *in-silico* bisulfite converted reference genome (BosTau8/BTau_4.6.1) with the Babraham Bismark software (v0.13.1, Babraham Bioinformatics, UK) using the *bismark_genome_preparation* command, the entire reference genome and with the *--non_directional* parameter applied. Babraham Bismark is designed for aligning bisulfite sequencing data while simultaneously making methylation calls. In the process of bisulfite sequencing, un-methylated cytosines are converted to uracil, while methylated cytosines are unaffected. The result of this is four sets of potential sequences at any given locus. To determine which of these four potential sequences is correct, Bismark creates two bisulfite converted reference genomes, one that is a C → T conversion and one that is a G → A conversion to account for conversion on the reverse strand. Each sequence read is also bisulfite converted *in silico* and is aligned to the pre-converted version of the reference genome. The best alignment is then identified and used to make a methylation call. Alignments that are uniquely mapped were kept for analysis. Alignments that mapped to multiple regions were discarded. Due to enrichment-constraints of RRBS libraries, duplicates measured as a result of shorter, overlapping paired end inserts were not removed in the sequence data and were counted as two reads.

On average, mapping efficiencies were 25% and ranged between 23-27% and the average number of unique CpGs identified were 6.0 million and ranged between 4.8

and 6.6 million. Differentially methylated CpGs falling within -2.5 kb, +1.0 kb of an annotated transcription start site (TSS) were defined as being within the promoter region of a gene. Methylation ratios were used as the comparison parameter to test statistical differences in DNA methylation and these were defined as the number of reads overlapping a particular CpG site, which contained either a cytosine or thymine nucleotide. Ratios were then calculated as Methylation Ratio (Mr) = (C)/(C + T).

### 2.3.6 Functional Analysis

KEGG (Kyoto Encyclopedia for Genes and Genomes) pathway, GO (Gene Ontology) and UCSC (University of California Santa Cruz) Transcription Factor Binding Site (TFBS) analyses were performed using the DAVID (the Database for Annotation, Visualization, and Integrated Discovery) 6.8 platform (Huang da et al., 2009b, a). Four lists of official gene IDs (all IDs or promoter only with ≥5X or ≥10X coverage) were generated for sites located within annotated genes that were hyper-methylated in either young or old cultures (Additional Files 1 and 2). A suggestive analysis was performed on all differentially methylated genes with ≥5X coverage and a conserved analysis was performed with only genes that had ≥10X coverage. Gene IDs from all three gene regions (exon, intron and promoter) were inputted into DAVID, converted to Entrez gene IDs, and default parameters were used for KEGG and GO analyses. Only those genes that were covered by RRBS, either at the 5X or 10X level, were set as the background. Promoter gene IDs were used in transcription factor enrichment analysis and TFBS analysis was similarly run with default parameters and *Homo sapiens* as the background due to a lack of support for *Bos taurus* within the UCSC_TFBS tool.

### 2.3.7 Gene Expression of Selected Genes

Selected differentially methylated genes and LPS response genes were measured in all fibroblast cultures exposed to LPS for 0, 2 and 8 hours. RNA was isolated using the 5 Prime PerfectPure™ RNA Cultured Cell Kit (5 Prime) per manufacturer's instructions and quantified using a Qubit™ 2.0 Fluorometer (Life Technologies, Carlsbad, CA). First strand cDNA synthesis was done using the ImpromII™ Reverse Transcriptase Kit (Promega, Madison, WI). To determine gene expression, quantitative reverse transcriptase PCR (RT-qPCR) was performed using Thermo Scientific™ Maxima™ SYBR™ Green/Fluorescein 2X qPCR Master Mix (Thermo Fisher) on a CFX96 Touch™ Real-Time PCR machine (Biorad, Hercules, CA) on the selected genes. The gene β-Actin was used as a housekeeping gene control. Cycling conditions were as follows: 95°C for 2 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute and 72°C for 1 minute after which a melt curve was inserted. All oligonucleotide primer sequences are shown in Table 2.1. Unless already published primers were available, all primers were designed using Primer 3 on NCBI. An amplicon of 200 – 300 base pairs was designed and the primer binding site could not contain any known SNP. Each primer sequence was then analyzed with an NCBI nucleotide BLAST search to ensure only the intended *Bos taurus* gene was detected. Melt curve analysis was performed on each primer pair and included a negative control reaction (no cDNA). Melt curve analysis revealed only one peak, indicative of a single product, with no peaks associated with the negative control.

### 2.3.8 Statistical Analysis

To assess differences in cytokine protein production, a paired Student's *t* test was performed using GraphPad Prism Version 6.0 for Windows (GraphPad Software, La Jolla, CA). Differences in DNA methylation at adequately covered (≥5X coverage based on similar RRBS studies (Pan et al., 2012, Begue et al., 2017)) CpG sites was performed at Zymo Research and determined using a paired Student's *t* test comparing methylation ratios (i.e. percent methylation of each fibroblast sample) at each site. Since all young and old cultures were individually sequenced, statistical analysis to determine significant differences in methylation were determined at a per individual basis taking into account variation within a sample and comparing each animal's young sample versus their old sample. A p-value of $<0.05$ was applied to assess significance. In addition, a cut-off of at least 25% difference in methylation was included when assessing gene expression levels as these regions are much more likely to have an influence on expression.

A clustering dendogram was made according to methylation ratio on the top 100 differentially methylated sites, based on p-value. The clustering analysis was performed at Zymo Research using a standard data analysis algorithm with a "complete" linkage method and a "Euclidean Distance" metric. Significance for GO, KEGG and TFBS were determined by a modified Fisher's Exact test in DAVID as previously described (Huang da et al., 2009b) and multiple comparisons correction were done using the Benjamini-Hochberg method. Gene expression differences were determined using either a paired (young vs. old) or unpaired (Angus vs. Holstein) Student's *t* test at each time point in GraphPad. Protein concentrations and delta Ct values are expressed as the mean +/- standard error (SEM).

## 2.4. Results

### 2.4.1 Fibroblast Cytokine Response to LPS

Dermal fibroblasts from the same six animals taken at 5 and 16 months of age were revived from cryopreservation for a total of 12 cultures (6 young vs. 6 old). Cultures were subsequently stimulated with LPS (100 ng/ml) for 36 hours to determine cytokine protein concentration in the media. Media from unstimulated cultures was used as a negative control. Protein concentrations of interleukin-8 (IL-8) are shown in figure 2.1, panel A. As expected, the 16-month cultures produced significantly more IL-8 ($P<0.01$) than the 5-month cultures. At 36 hours, older cultures had an approximately 5-fold higher concentration of IL-8 than young cultures. No detectable IL-8 was produced in media only controls. Interleukin-6 (IL-6) was also measured in media post 36-hour LPS stimulation. In line with IL-8 protein, 16-month cultures produced significantly more IL-6 ($P<0.01$) than 5-month cultures (Figure 2.1, panel B). This difference in older cultures was about 2.5-fold more IL-6 than young cultures. Again, control media IL-6 levels fell below detection limits of this assay.

Next, to determine if cytokine gene expression was also different between the young and old cultures, RT-qPCR was performed on fibroblasts at 0, 2 and 8 hours post LPS stimulation. In line with protein production, *IL8* and *IL6* gene expression levels were upregulated ($P<0.01$) in old versus young cultures (figure 2.1, panels C and D). Older fibroblast cultures had a 15.9 and 10.9-fold increase in *IL8* gene expression and a 7.3 and 13.4-fold increase in *IL6* gene expression over younger cultures at 2 and 8 hours post LPS stimulation, respectively.

Cytokine protein and expression analyses confirmed that the older dermal fibroblast cultures were much more responsive to LPS than younger cultures. Genomic DNA was then isolated from unstimulated cultures, grown from the same cryopreserved stock used to assess LPS response, and analyzed by RRBS to determine whether methylation differences may contribute to differences in the LPS-induced response.

### 2.4.2 Reduced Representation Bisulfite Sequencing in 5-month and 16-month Dermal Fibroblasts

As outlined in the RRBS project workflow in figure 2.2, genomic DNA from all the cultures were treated with a methylation insensitive restriction enzyme *MSP*I. The restriction enzyme cuts at CCGG sites, following the first cytosine, yielding CG sites on both ends of the resulting DNA fragments. This results in enrichment of fragments from regions in the genomic DNA with many CpG sites, such as CpG islands, that are typically associated with promoter regions of DNA (Deaton and Bird, 2011). In our analysis, 65% of annotated promoter regions, defined as -2500 to +1000 bp from the transcription start site (TSS), had greater than 50X coverage, 8% had less than 50X but greater than 10X coverage, 4% had less than 10X coverage, and 23% were not represented.

Following bisulfite conversion, sequencing, and post-sequencing bioinformatics, a paired T-test was performed on the methylation ratios between young and old cultures at each sequenced CpG site that met coverage requirements. Methylation analysis revealed 14,094 differentially methylated CpG sites with $P<0.05$ and at least 5X coverage. Of these, 9,351 (67%) were more methylated in young cultures and 4,743 (33%) were more methylated in old cultures. Clustering analysis was performed on the

top 100 differentially methylated sites (i.e. sites with the greatest significant difference in methylation ratios). The old cultures and young cultures clustered separately, indicating that two distinct methylation profiles exist, where young cultures form one profile and old cultures form a second (Additional File 3).

### 2.4.3 Suggestive Analysis of Differentially Methylated Genes

Next, we performed a comprehensive, but suggestive functional analysis of all the sites that fell within gene regions and had ≥5X coverage. Of the 9,351 sites more methylated in young cultures, 752 regions were located in annotated promoters, 720 in gene exons, and 1926 in gene introns. Of the 4,743 sites more methylated in old cultures, 365 were located in annotated promoters, 337 in gene exons, and 965 in gene introns. Sites that fell within annotated genes were used to generate lists of gene IDs with greater methylation in either young or old cultures and functional analysis was performed using DAVID. KEGG pathway analysis on genes more methylated in young cultures identified 5 significantly enriched pathways. These included the "Adherens Junction", "Proteoglycans in Cancer", "Rap1 Signaling Pathway", "Pathways in Cancer" and "Melanogenesis". It is worth noting that the "PI3K-Akt Signaling Pathway" was also enriched with P=0.06 after multiple comparisons correction. In older cultures, 16 pathways were significantly enriched, with the "Cholinergic Synapse" pathway as the top result. A number of secretory pathways and hormone synthesis pathways were also significantly enriched. Among the 16 pathways, those that were of most interest to the current study were the "Calcium Signaling Pathway", "Inflammatory Mediator Regulation of TRP Channels" and "cAMP Signaling Pathway". Among the top significantly enriched GO terms in genes with greater methylation in young cultures were

"Transcription Repressor Activity", "Sequence Specific DNA Binding", and "RNA Polymerase II Regulatory Region Sequence-Specific DNA Binding". Interestingly, in older cultures, only one GO term, "Plasma Membrane" was significant and, in contrast to young cultures, lacked terms related to regulation of gene transcription.

Using the annotated genes from only the promoter sites with greater methylation in either young or old cultures, a transcription factor enrichment analysis was performed using DAVID and *Homo sapiens* as the background species, because *Bos taurus* is not yet supported in the UCSC_TFBS function. Table 2.2 shows the top 15 most significantly enriched transcription factors that regulate the inputted gene promoters more methylated in young cultures and Table 2.3 shows transcription factors enriched in gene promoters more methylated in old cultures. A number of transcription factors associated with immune system regulation and the inflammatory response were associated with promoters more methylated in both young and old cultures. Most noteworthy in young cultures was greater methylation in NF-κB associated genes. Also, significantly more methylated in young cultures were genes regulated by PAX5 (also known as B-cell activating protein), and CREB (cAMP-regulated binding protein), a phosphorylation responsive transcription factor that binds to cAMP responsive elements.

In old cultures, PAX5 was similarly enriched along with SP.1, p300 (a CREB co-activator), and ATF or activating transcription factors, which are diverse members of the ATF/CREB family of transcription factors with a range of physiological functions.

### 2.4.4 Conserved Analysis of Differentially Methylated Genes

In a second, conserved analysis we focused solely on sites with ≥10X coverage, which is the minimum coverage that is necessary to accurately determine differentially

methylated sites in an RRBS analysis. The additional filtering left 2,865 differentially methylated sites. Of these, 1,922 were more methylated in young cultures and 943 were more methylated in old cultures. In young cultures, 134 gene promoters, 98 exons, and 354 introns were hyper-methylated, and in old cultures only 42 gene promoters, 45 exons, and 150 introns were hyper-methylated in comparison to young cultures. The genes associated with these regions were used to conduct a conserved GO, KEGG and TFBS analysis. Interestingly, with the additional filtering we found no significant GO terms associated with either young or old cultures, although "Semaphorin Receptor Activity" trended ($P<0.10$) towards significance in old cultures. Two KEGG pathways, "Adherens Junction" and "Proteoglycans in Cancer" remained significantly associated with young cultures and no KEGG pathways were found to be associated with old cultures. Finally, 21 transcription factors were identified as significantly enriched in hyper-methylated promoter regions in young cultures, the top 10 of which are listed in table 2.4. We did not identify any transcription factors enriched in our assessment of hyper-methylated promoters in old cultures. Many of the transcription factors that were identified in the suggestive analysis in young cultures remained significant, including PAX5 and most notably, NF-κB that was found to be associated with 56 out of 134 gene promoters more methylated in young cultures.

### 2.4.5 Fibroblast Gene Expression of Differentially Methylated Genes

Selected differentially methylated genes were analyzed by RT-qPCR to determine whether changes in methylation resulted in differences in gene expression. The rationale for choosing the analyzed genes was that there was a greater than 25% methylation difference in at least one region in young versus old cultures, the sites had on

average greater than 10X coverage across the 12 animals, methylation was greater in young versus old cultures and, there was some evidence in the literature that the genes are involved in immune response regulation. The six selected genes, and number and location of differentially methylated regions, are listed in Table 2.5, along with a summary of expression differences.

Gene expression analysis of the LPS responsiveness of the six selected genes hyper-methylated in young versus old cultures is shown in Figure 2.3. Overall, 5 of the 6 selected genes showed a reduced expression in cultures established from the animals at 5 vs. 16 months of age. Two of the 6 genes, *FES* and *PIK3R1*, had reduced expression in the younger cultures at all 3 time points, with a 5.7, 4.3, and 6.3-fold ($P<0.01$) lower expression in *FES* and 3.0, 2.6, and 2.5-fold ($P<0.01$) lower expression in *PIK3R1* at 0, 2, and 8 hours post LPS stimulation, respectively. Both genes had differentially methylated sites located in the promoter region, a single site located 23 base pairs upstream from the transcription start site (TSS) of *PIK3R1* and two sites, 4 and 6 base pairs, downstream of the TSS in *FES*. Additionally, *FES* had two differentially methylated sites located within exon regions and, *PIK3R1* had one site located within the first intron of the gene. Expression of these genes was not affected by LPS stimulation.

A gene that encodes a TNF superfamily member ligand, *TNFSF13* also known as APRIL, also showed significantly lower expression in young cultures at 0 and 8 hours post LPS stimulation (2.0 and 2.8-fold, $P<0.05$), with a trend towards significantly lower expression at 2 hours (1.6-fold, $P<0.10$), but was not affected by LPS stimulation. *TNFSF13* had one differentially methylated site in the gene promoter region, 145 bases upstream from the TSS in the anti-sense position and directly downstream from a site on

the positive strand with 93% sequence similarity to the consensus NF-κB binding site. The gene, *NFATC1*, which encodes an LPS responsive transcription factor, had significantly lower expression in young versus old cultures at 0 and 2 hours post LPS stimulation, with both time points showing a 1.8-fold ($P<0.05$) lower expression in young cultures. Expression of *NFATc1* also increased 17.5 +/- 5.6-fold 2 hours post LPS stimulation, with no difference in fold change between young and old cultures. By 8 hours post-challenge, expression returned to near basal levels. The *NFATC1* gene had one differentially methylated site located within a CpG island in the promoter region, approximately 1000 bp upstream from the TSS and downstream of a potential Sp1 binding site. Two additional differentially methylated sites were located within the last intron of the *NFATC1* gene.

The only selected gene that did not have differences in promoter methylation, *RORA*, had 6 differentially methylated sites located in the first intron of the gene. Gene expression analysis showed significantly lower levels of expression in young versus old cultures at 8 hours post LPS treatment (2.1-fold, $P<0.001$) with no change in response to LPS. Finally, we measured *TCF7*, the gene that transcribes the transcription factor TCF7, that had 2 differentially methylated sites located within a CpG island in the promoter region. The first CpG was located approximately 1200 bp upstream from the TSS and near to a potential NFAT5 binding site, and the second CpG was approximately 979 bp upstream from the TSS on the anti-sense strand. Interestingly, no significant expression differences were detected in young versus old cultures. However, there was a small increase in gene expression of *TCF7* in response to LPS. Expression was increased

2.3 +/- 0.7-fold and 2.7 +/- 0.9-fold at 2 and 8 hours, respectively, with no difference between young and old cultures.

### 2.4.6 Gene Expression of Differentially Methylated Genes in Angus vs. Holstein Fibroblast Cultures

Finally, we wanted to determine whether differentially methylated genes identified in young versus old cultures would differ in expression in a second high vs. low LPS responsive cattle phenotype recently discovered in our laboratory. Just as with the age difference, we have shown that dermal fibroblasts collected from the Holstein breed are much more responsive to LPS than fibroblasts collected from the Angus breed (Benjamin et al., 2016a). To determine whether similar gene expression differences could be detected between breeds, RT-qPCR was performed on age-matched dermal fibroblast cultures at 0, 2 and 8 hours post-LPS treatment. Greater LPS-induced IL-8 and IL-6 protein production and gene expression in Holstein vs. Angus cultures have been described elsewhere (Benjamin et al., 2016a).

Gene expression analysis was performed on the same 6 genes that were differentially methylated in the young versus old fibroblast cultures, where young cultures had greater methylation and, with the exception of *TCF7*, lower expression. As shown in figure 2.4 and summarized in Table 2.5, the majority of the genes were similarly upregulated in Holstein cultures as they were in older cultures. Two notable exceptions to the pattern seen in our young versus old analysis were in *TNFSF13*, where no difference was measured in Angus versus Holstein cultures, and in *TCF7*, which was upregulated in Holstein cultures at 0 and 8 hours post LPS treatment. Under basal conditions, Holstein cultures expressed *TCF7* 4.5-fold ($P<0.01$) higher than Angus

cultures and at 8 hours post LPS *TCF7* expression was 4.3-fold ($P<0.05$) higher in Holstein cultures. Interestingly, at 2 hours post LPS treatment, Angus cultures increased *TCF7* gene expression 5-fold, while Holstein cultures only increased expression 2-fold. This fold increase at 2 hours was significantly higher in Angus cultures ($P<0.05$) as compared to Holstein cultures, although Holstein cultures did have a significantly ($P<0.05$) higher level of expression at 2 versus 0 hours. While Holstein cultures continued to maintain elevated expression of *TCF7*, Angus cultures had started to return to basal levels of expression at 8 hours post LPS, although expression was still significantly higher ($P<0.05$) than without treatment.

Similar to old cultures, Holstein cultures had significantly ($P<0.05$) higher expression of *FES* at 0 and 2 hours post LPS treatment, with expression levels being 3.7 and 5.4-fold higher, respectively, and a trend (4.8-fold, $P<0.10$) towards higher expression in Holstein cultures at 8 hours post LPS. *PIK3R1* also had higher levels of expression ($P<0.01$) 2 hours post-LPS treatment in Holstein cultures. This difference was due to a significant ($P<0.01$) 4-fold decrease in *PIK3R1* gene expression in Angus cultures that was not detected in Holstein cultures. By 8 hours post LPS treatment, *PIK3R1* expression returned to near-basal levels in Angus cultures.

Most noteworthy was the gene *NFATC1*, which showed a very similar gene expression pattern between breeds as was seen in the age difference. Both Angus and Holstein cultures significantly increased their expression of *NFATc1* at 2 hours post LPS treatment (30-fold and 12-fold respectively), with Holstein cultures having a 6.5-fold higher ($P<0.01$) level of expression over Angus cultures at 2 hours post LPS. Holstein cultures also trended towards greater expression (2.2 and 1.7-fold, $P<0.10$) at 0 and 8

hours post-LPS. By 8 hours post LPS, both breeds returned to basal levels of expression. Finally, *RORA* had higher ($P<0.01$) levels of expression in Holstein cultures at 0 and 2 hours post LPS treatment. At 0 hour, Holstein cultures had a 2.2-fold higher level of expression and at 2 hours they had a 2.0-fold higher level of expression. No LPS treatment effect was detected.

## 2.5. Discussion

An increase in the systemic production of pro-inflammatory mediators has been well characterized as a component of the aging process, heightening the risk of cardiovascular, neurodegenerative and other diseases in humans. This process, coined by Franceschi et al.,(Franceschi et al., 2000) as "inflamm-aging", is highlighted by increased concentrations of serum TNF-α, IL1-ß and IL-6 along with acute phase proteins, such as C reactive protein (Franceschi et al., 2000, Baylis et al., 2013). To determine the potential mechanism behind an increase in chronic inflammation associated with age, both in healthy aging individuals and during the diseased state, regulation of pro-inflammatory gene expression by DNA methylation has been widely studied.

A substantial decrease in methylation at a single CpG site in the promoter of the *IL6* gene was measured in patients with rheumatoid arthritis versus healthy controls. In the same study, ranking of healthy control macrophages in response to LPS showed that an increase in LPS-induced IL-6 production was correlated with a decrease in *IL6* promoter methylation (Nile et al., 2008). Epigenetic regulation of another pleiotropic pro-inflammatory cytokine gene, *TNF,* has been similarly described. In comparison to healthy controls, patients with previous exposure to the Dengue virus have higher blood *TNF-α* expression with a concomitant decrease in promoter methylation (Gomes et al., 2016).

DNA methylation changes have also been reported to occur in *TNF* in humans as part of the natural aging process. Macrophages obtained from 78 healthy individuals showed a 1.4% per decade decrease in three 5' CpG motifs located within the *TNF* promoter (Gowers et al., 2011). Increased methylation of *TLR4*, the key TLR in response to LPS, has also been shown in the context of intestinal epithelial homeostasis, whereby intestinal epithelial cells are made tolerant to stimulation by commensal microbiota through down-regulation of TLR4 to avoid continuous inflammation (Takahashi et al., 2009). In that study, a comparison between a high responding and a low responding intestinal epithelial cell line showed that the lower responding cells not only exhibit decreased expression but also enhanced methylation in 11 CpG sites in the gene promoter of the *TLR4* gene. Furthermore, inhibition of DNA methylation with AZA treatment in these two cell lines abolished the difference in *TLR4* expression, further establishing a causal role for DNA methylation in the regulation of TLR4 (Takahashi et al., 2009).

Research on epigenetic regulation of the immune response in livestock species has been somewhat more limited. However, it has been shown that in response to LPS treatment, bovine PBMCs decrease expression of methylation and histone deacetylation enzymes, suggesting LPS treatment may result in lower gene methylation and increased histone acetylation (Doherty et al., 2013). The effects of *Mycobacterium bovis* infection on global DNA methylation and gene expression have also been reported in bovine CD4+ T-cells, whereby, DNA methylation was negatively correlated with expression of interferon-γ (*IFNG*) and a gene that encodes the OX-40 receptor (*TNFRSF4*). The two genes, *IFNG* and *TNFRSF4*, are involved in T-cell response and proliferation, respectively, and provide one potential mechanism that contributes to the shift from Th1

to Th2 T-cell response following bovine tuberculosis infection (Doherty et al., 2016). Epigenetic mechanisms have also been shown to play a role in the hepatic expression of *TLR4*, lipopolysaccharide binding protein (*LBP*) and haptoglobin in response to *E. coli* mastitis. Chromatin de-compaction and increased expression of key innate response genes, *LBP, HP, TLR2* and *TLR4* occurs in liver biopsies following an intra-mammary challenge with *E. coli*. In *TLR4,* the chromatin changes are also correlated with promoter de-methylation (Chang et al., 2015a). In a similar study that assessed the effects of feeding a high concentrate diet to induce sub-acute ruminal acidosis (SARA), chromatin de-compaction and expression of these innate response genes was again increased in liver biopsies with subsequent DNA de-methylation seen in all four genes. Presumably, these changes were a result of greater concentrations of LPS found in the rumen, and hepatic and portal veins of cows experiencing SARA (Chang et al., 2015b).

To elucidate the underlying causes of differences in innate response to mastitis causing pathogens, we have taken advantage of two phenotypes with clear response differences, age and breed, where fibroblasts from older versus younger animals (Green et al., 2015), and Holstein versus Angus animals (Benjamin et al., 2016a), have much higher *in vitro* responses. The age-dependent difference in response has also been shown to be at least partially mediated by epigenetic mechanisms. Inhibition of DNA methylation and histone deacetylation in fibroblasts isolated from the same animal at 5- and 16-months of age abolishes differences in *in vitro* cytokine production, suggesting methylation, chromatin modifications, or both contribute to the age-dependent increase in response (Green and Kerr, 2014). To determine which genes are regulated by DNA methylation, methylated CpG island recovery assay or MIRA-seq, was previously

performed on both the age and breed phenotypes following RNA-seq analysis. Unfortunately, this technique, which utilizes the protein complex MBD2b-MBD3L1 complex to enrich for methylated regions within the genome, has only moderate resolution (~500 bp), and few differences were detected in both phenotypes that fell within gene regions, while none of these differences were detected in regions with a role in the innate response. Since it is known that small changes in methylation can be effective in changing gene expression, it was necessary to utilize a more sensitive technique, such as was performed in the current study.

### 2.5.1 Methylation Differences in 5- versus 16-month Dermal Fibroblasts

Reduced representation bisulfite sequencing was performed on fibroblasts taken from 5- and 16-month old heifers that had significantly different IL-8 and IL-6 protein production and gene expression. This analysis revealed 14,094 differentially methylated CpG sites. Approximately two-thirds of these sites were more methylated in younger cultures, consistent with previous data suggesting global hypo-methylation in older individuals (Bacalini et al., 2014, Marttila et al., 2015). The transcription factor, NF-κB, which is a major activator of pro-inflammatory gene expression downstream of a number of pattern recognition receptors and TNF-associated receptors (van Delft et al., 2015), was identified as significantly associated with hyper-methylated promoter regions in young cultures. This suggests that increased methylation in young cultures may block NF-κB binding sites, reducing transcription of pro-inflammatory genes. Blocking of NF-κB gene transcription by DNA methylation has been shown to occur in patients with secondary acute myeloid leukemia (sAML), where hematopoietic progenitor cells fail to express Fas receptor, an activator of apoptosis. The *FAS* gene was shown to contain 3

canonical NF-κB binding sites and increased methylation in bone marrow blast cells from patients with sAML as compared to those with low-risk myelodysplastic syndrome whose Fas expression remains intact. In addition, re-expression of Fas occurred in sAML patients upon de-methylation with AZA (Ettou et al., 2013). Although only identified in our suggestive (≥5X coverage) analysis, the transcription factor, cAMP regulated binding protein (CREB), was also associated with gene promoters more methylated in young cultures. Downstream of a wide variety of serine-threonine kinases (such as PKA, PKC, and p38 MAPK) CREB regulates a multitude of processes, including the innate immune response (Wen et al., 2010). Recently, CREB has been shown to mediate TNF-α dependent GM-CSF production in primary asthmatic lung fibroblasts and human fetal lung fibroblasts (Koga et al., 2016). CREB also increases LPS and TLR4 dependent IL-6 production in vascular smooth muscle cells which may contribute to the vascular inflammation seen in atherosclerosis (Lee et al., 2016).

In older cultures, transcription factor binding sites for Sp1, p300, and ATF were enriched in hyper-methylated genes. Numerous genes contain Sp1 binding sites, which are located in GC-rich regions often regulated by DNA methylation. Somewhat contradictory to our hypothesis, Sp1 does up-regulate expression of some innate response genes. In intestinal epithelial cells, basal expression of *TLR5* is regulated by Sp1. Interestingly, inducible expression of *TLR5* is also partially mediated by p300, in agreement with our results that both Sp1 and p300 regulated genes were hyper-methylated in older cultures (Thakur et al., 2016). Alternatively, in response to LPS, Sp1 enhances macrophage transcription of IL-10, a key anti-inflammatory cytokine (Wang et al., 2014). ATFs are members of the activating transcription factor/cAMP responsive

element binding protein (ATF/CREB) family of transcription factors. It was not clear with DAVID analysis which of the family members were enriched in regulation of the genes more methylated in older cultures, however, analysis of just the 106 genes regulated by ATF showed that 62 were regulated by ATF6. ATF6 is a key transcription factor that is activated under ER stress and mediates the unfolded protein response. Again, contrary to our data, ER stress contributes to inflammation during an infection with *Brucella abortis* in a NOD1/NOD2 dependent manner, and the unfolded protein response has been shown to strengthen NF-κB dependent inflammation (Mohammed-Ali et al., 2015, Keestra-Gounder et al., 2016). However, other ATFs, such as ATF3, have been shown to have the opposite effect and reduce inflammatory gene expression (Labzin et al., 2015). Furthermore, when additional stringency was applied to the TFBS analysis to include only genes that contained differentially methylated sites with ≥10X coverage, we failed to identify any transcription factors associated with genes hyper-methylated in old cultures, unlike what was found in young cultures. An additional caveat to the transcription factor binding data used in the current study is that conclusions were drawn on sequence conservation found between human and rodent alignments. Sequence conservation does not necessarily result in transcription factor binding and regulation and whether enrichment of the identified transcription factors regulate cellular response differences to LPS in a methylation-dependent manner would need to be validated in our model.

### 2.5.2 Gene Expression Analysis of Differentially Methylated Genes

Methylation of DNA is most known for its role in repressing gene transcription. To determine if the measured methylation differences had any effect on gene expression,

we chose six genes that exhibited higher methylation in young cultures for RT-qPCR analysis. We hypothesized these six genes would have lower expression in the young cultures due to their increased methylation status. We chose the following six genes for their potential role in the innate response: *TNFSF13, PIK3R1, NFATC1, FES, TCF7* and *RORA*. The TNF superfamily 13, or APRIL, protein is a surface or secreted ligand recognized by the transmembrane activator and calcium-modulating cyclophilin ligand interactor (TACI) and B-cell maturation antigen (BCMA) receptors and has been implicated in the pathogenesis of numerous pro-inflammatory conditions such as rheumatoid arthritis (Weldon et al., 2015), atherosclerosis (Sandberg et al., 2009), lupus (Salazar-Camarena et al., 2016) and psioriasis (Alexaki et al., 2012). Moreover, APRIL has been shown to activate NF-κB dependent cytokine production in macrophages (Lee et al., 2011) and keratinocytes (Alexaki et al., 2012). In our analysis, fibroblast expression was significantly up-regulated in older cultures at 0 and 8 hours post LPS treatment as we expected. The single site hyper-methylated in young cultures was located near to the consensus NF-κB binding sequence, 5′-GGGRNYY YCC-3′. However, since expression of the gene did not increase after LPS treatment and was already up-regulated under basal conditions, it is unlikely NF-κB regulates its transcription in response to LPS.

The gene, *PIK3R1*, transcribes the p85α subunit of phosphoinositide 3-kinase (PI3K), a lipid kinase that phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3). The p85α subunit is a regulator of kinase activity, whereby it is thought to inhibit PI3K activity under most circumstances (Cheung et al., 2015). Interestingly, in a murine model of polymicrobial sepsis, inhibition of the PI3K pathway increased serum IL1-β, IL-6 and TNF-α levels, and decreased the

survival rate of septic mice (Williams et al., 2004). Alternatively, pharmacological activators of PI3K signaling have recently been suggested as anti-inflammatory drug candidates (Kok and Saez, 2014). However, the role of PI3K in inflammation is complex, since kinase activity can also activate leukocytes and is further complicated by the existence of multiple isoforms of PI3K with a variety of physiological functions (Hawkins and Stephens, 2015). In our model, *PIK3R1* expression was up-regulated in older cultures at all 3 time points post LPS with a fairly consistent fold change of 2.5 – 3.0-fold. A single CpG site in the promoter region was more highly methylated, only 23 base pairs from start site of the gene, potentially interfering with the progression of the RNA polymerase enzyme complex.

Nuclear factor of activated T-cells, calcineurin dependent 1 (NFATc1), the gene product of *NFATC1,* is a calcium dependent transcription factor that is necessary for lymphocyte development and is expressed in a number of other cell types such as dendritic cells (Zanoni et al., 2009), macrophages (Yarilina et al., 2011) and endothelial cells (Suehiro et al., 2010), and is responsible for mediating TLR4 independent, CD14 dependent pro-inflammatory gene expression in response to microbial agonists, such as LPS. Interestingly, the *NFATC1* gene is less methylated and more expressed in individuals with lower socioeconomic status which is a known risk factor for increased inflammation that can lead to pathologies such as type II diabetes (Stringhini et al., 2015, Stringhini et al., 2016). In our study, *NFATc1* was induced following LPS stimulation in both young and old fibroblasts, further solidifying its role as an LPS-responsive transcription factor. In the promoter, the differentially methylated CpG was located directly downstream of a Sp1 binding site that has been shown in numerous recent studies

to bind at lower levels with increased DNA methylation status (Gopisetty et al., 2013, Zhang et al., 2014, Cai et al., 2016). In addition, two other sites were located in the last intron of the gene. This is of interest because an enhancer element within the last intron of the *NFATc1* gene that activates transcription to an even greater extent than the promoter region of the gene has been identified using human lymphocytes (Rudolf et al., 2014).

Feline sarcoma oncogene (FES) is a non-receptor tyrosine kinase that, in humans, stimulates hematopoiesis, osteoclastogenesis, and mast cell activation (Hellwig et al., 2012). It is worth mentioning that both FES and PI3K activate mast cells through a similar SCF-c-Kit-integrin pathway, and the two proteins have been speculated to interact with one another through tyrosine phosphorylation of PI3K by FES (Tan et al., 2003, Smith et al., 2010). As with *PIK3R1*, *FES* kinase expression was also similarly up-regulated at all time points in older cultures while younger cultures had two hyper-methylated sites near to the transcriptional start site which again may interfere with transcriptional elongation by RNA polymerase.

Transcription factor 7 (TCF7), transcribed from the gene *TCF7,* is a transcription factor that is crucial to the proper development of T-cells in the thymus, but has been shown to inhibit the development of regulatory T-cells, or Tregs, which suppress inflammation and aid in the resolution of infection (Barra et al., 2015). Although several methylation differences were detected within the promoter region of *TCF7*, no difference in expression was measured in fibroblasts either basally or post LPS. There are a couple of possible explanations for this discrepancy. Methylation itself may not be sufficient to repress expression and it is likely an over-simplification to assume

methylation always leads to repression. For example, in a study assessing the relationship between methylation and gene transcription in the blood of 148 healthy human subjects, the authors found that 276 of 798 local associations were actually positively associated, where less methylation led to less transcription or more methylation led to more transcription (van Eijk et al., 2012). Other factors, such as histone modifications and transcription factors that also have an effect on transcription should be taken into consideration as they can override any differences in methylation as has been shown in the differentially imprinted gene *IGF2R* (Vu et al., 2004). Lastly, it is possible that splice variants of *TCF7* exist that exhibit differential expression in our samples but were not measured in our RT-qPCR analysis.

Finally, RORA, or retinoic acid related (RAR) orphan receptor alpha, belongs to a family of nuclear receptor transcription factors (RORs) that have known roles in the innate and adaptive immune response, circadian rhythm, and metabolic regulation, among others (Cook et al., 2015). Specifically, RORA is required for the development of type II innate lymphoid cells and mice lacking the gene experience reduced eosinophil-mediated lung inflammation in response to allergens (Halim et al., 2012) and are less susceptible to obesity induced inflammation in response to a high fat diet (Kang et al., 2011). We detected a small, but significant increase in *RORA* expression in older fibroblasts at 8-hours post LPS. In this case, we identified 6 methylation differences all within first intron of the gene, which can contain enhancer elements for gene activation (Blattler et al., 2014).

### 2.5.3 Expression of Differentially Methylated Genes in Angus versus Holstein Fibroblasts

In our final experiment, we wanted to determine whether similar expression differences could be detected in another high-low response phenotype, a breed difference identified in our lab that shows the Holstein breed having a much higher *in vitro* response as compared to the Angus breed (Benjamin et al., 2016a). Genes similarly up-regulated in Holsteins might suggest a conserved mechanism of regulation, such as DNA methylation or other epigenetic modifications. This of particular interest in the breed difference as these two breeds are not only genetically selected for divergent purposes but also experience highly dissimilar neonatal environments, leaving room for possible environmental interventions. Many studies have shown that neonatal nutrition, maternal care, and exposure to immune agonists can shape an individual's adult epi-genotype, and all three of these environmental influences differ between Holstein and Angus calves. Angus calves are maternally cared for in a pasture setting, drink milk from their dam, and are exposed to a variety of maternally-derived commensal and environmental microbes in contrast to Holstein calves that are raised individually without maternal contact, fed milk replacer, and are housed in a considerably more defined environment.

Expression analysis of fibroblasts isolated from the two breeds showed some similar differences to our age-dependent differences. In the high responding Holstein breed, *FES, NFATC1, RORA,* and to a lesser extent *PIK3R1,* were up-regulated. Additionally, *TCF7* was up-regulated, a difference not detected in the young-old culture comparison. This discrepancy may indicate a genetic difference as a result of selection for different traits (beef versus dairy) between Angus and Holstein breeds. No difference

was measured in *TNFSF13,* suggesting the loss of methylation is unique to the aging process. Most noteworthy of the differentially expressed genes were *FES* and *NFATC1*. These two genes showed an identical pattern of up-regulation in Holstein cells as was seen in older cells, indicating they may be similarly regulated. However, additional experiments with the Holstein and Angus cultures would be necessary to confirm whether expression differences are due to differences in methylation or other epigenetic factors, genetic differences, or a combination of factors. Expression of *FES* is up-regulated basally in both high response phenotypes, making it a candidate biomarker for selection strategies, which would require a rapid test without the need of culturing cells and treating with LPS. Additionally, *NFATC1* is of interest due to its role in the response to LPS and as a known contributor to many pro-inflammatory pathologies. Future studies assessing methylation differences in Angus and Holstein breeds are underway and should provide insight into how this phenotype is regulated.

One limitation of the current study was that the RRBS technique does not allow for a comprehensive methylation analysis and as a result many CpG sites are missed. This may be one reason why we failed to identify methylation differences in classic LPS response genes that show similar up-regulation of expression in both high responding phenotypes. Targeted bisulfite sequencing of candidate genes, such as *TLR4* and *IL8*, will be the subject of future experiments to determine whether methylation may influence expression of genes that directly mediate LPS response.

## 2.6. Conclusions

Overall, the current study has shown that many sites differ in their methylation status in young versus old cultures, with the majority of sites becoming less methylated

as the animals increased in age. Candidate innate response genes that were hyper-methylated in young cultures also exhibited lower expression in most instances, indicating methylation may suppress expression in younger cultures making them less responsive to TLR agonists, such as LPS. Expression analysis in a second high-low response phenotype, Angus versus Holstein fibroblasts, identified two genes (*FES* and *NFATC1*) that showed an identical response pattern as the young and old cultures. These two genes may be useful as candidate biomarkers for predicting whether an animal will have a high or low response phenotype, and subsequently, whether she is susceptible to severe mastitis. In conclusion, knowledge of critical changes in methylation that increases the magnitude of inflammatory response within an individual may allude to the mechanisms of between-individual variation in the innate response. This is particularly important in diseases where an unregulated innate response contributes to the pathology of the disease, such is the case in bovine mastitis.

## 2.7 References

Alexaki, V. I., V. Pelekanou, G. Notas, M. Venihaki, M. Kampa, V. Dessirier, S. Sabour-Alaoui, E. N. Stathopoulos, A. Tsapis, and E. Castanas. 2012. B-cell maturation antigen (BCMA) activation exerts specific proinflammatory effects in normal human keratinocytes and is preferentially expressed in inflammatory skin pathologies. Endocrinology 153(2):739-749.

Bacalini, M. G., S. Friso, F. Olivieri, C. Pirazzini, C. Giuliani, M. Capri, A. Santoro, C. Franceschi, and P. Garagnani. 2014. Present and future of anti-ageing epigenetic diets. Mech Ageing Dev 136-137:101-115.

Ballou, M. A. 2012. Growth and Development Symposium: Inflammation: Role in the etiology and pathophysiology of clinical mastitis in dairy cows. Journal of animal science 90(5):1466-1478.

Barra, M. M., D. M. Richards, J. Hansson, A. C. Hofer, M. Delacher, J. Hettinger, J. Krijgsveld, and M. Feuerer. 2015. Transcription Factor 7 Limits Regulatory T Cell Generation in the Thymus. J Immunol 195(7):3058-3070.

Baylis, D., D. B. Bartlett, H. P. Patel, and H. C. Roberts. 2013. Understanding how we age: insights into inflammaging. Longev Healthspan 2(1):8.

Begue, G., U. Raue, B. Jemiolo, and S. W. Trappe. 2017. DNA Methylation Assessment from Human Slow- and Fast-Twitch Skeletal Muscle Fibers. J Appl Physiol (1985):jap 00867 02016.

Benjamin, A. L., B. B. Green, B. A. Crooker, S. D. McKay, and D. E. Kerr. 2016. Differential responsiveness of Holstein and Angus dermal fibroblasts to LPS challenge occurs without major differences in the methylome. BMC Genomics 17(1):258.

Benjamin, A. L., B. B. Green, L. R. Hayden, J. W. Barlow, and D. E. Kerr. 2015. Cow-to-cow variation in fibroblast response to a toll-like receptor 2/6 agonist and its relation to mastitis caused by intramammary challenge with Staphylococcus aureus. Journal of dairy science.

Blattler, A., L. Yao, H. Witt, Y. Guo, C. M. Nicolet, B. P. Berman, and P. J. Farnham. 2014. Global loss of DNA methylation uncovers intronic enhancers in genes showing expression changes. Genome Biol 15(9):469.

Cai, D., M. Yuan, H. Liu, Z. Han, S. Pan, Y. Yang, and R. Zhao. 2016. Epigenetic and SP1-mediated regulation is involved in the repression of galactokinase 1 gene in the liver of neonatal piglets born to betaine-supplemented sows. Eur J Nutr.

Callaway, J. B., S. A. Smith, K. P. McKinnon, A. M. de Silva, J. E. Crowe, Jr., and J. P. Ting. 2015. Spleen Tyrosine Kinase (Syk) Mediates IL-1beta Induction by Primary Human Monocytes during Antibody-enhanced Dengue Virus Infection. J Biol Chem 290(28):17306-17320.

Chang, G., W. Petzl, J. Vanselow, J. Gunther, X. Shen, and H. M. Seyfert. 2015a. Epigenetic mechanisms contribute to enhanced expression of immune response genes in the liver of cows after experimentally induced Escherichia coli mastitis. Vet J 203(3):339-341.

Chang, G., K. Zhang, T. Xu, D. Jin, J. Guo, S. Zhuang, and X. Shen. 2015b. Epigenetic mechanisms contribute to the expression of immune related genes in the livers of dairy cows fed a high concentrate diet. PLoS One 10(4):e0123942.

Cheung, L. W., K. W. Walkiewicz, T. M. Besong, H. Guo, D. H. Hawke, S. T. Arold, and G. B. Mills. 2015. Regulation of the PI3K pathway through a p85alpha monomer-homodimer equilibrium. Elife 4:e06866.

Cook, D. N., H. S. Kang, and A. M. Jetten. 2015. Retinoic Acid-Related Orphan Receptors (RORs): Regulatory Functions in Immunity, Development, Circadian Rhythm, and Metabolism. Nucl Receptor Res 2.

De Nardo, D. 2015. Toll-like receptors: Activation, signalling and transcriptional modulation. Cytokine 74(2):181-189.

Deaton, A. M. and A. Bird. 2011. CpG islands and the regulation of transcription. Genes & development 25(10):1010-1022.

Doherty, R., C. O'Farrelly, and K. G. Meade. 2013. Epigenetic regulation of the innate immune response to LPS in bovine peripheral blood mononuclear cells (PBMC). Vet Immunol Immunopathol 154(3-4):102-110.

Doherty, R., R. Whiston, P. Cormican, E. K. Finlay, C. Couldrey, C. Brady, C. O'Farrelly, and K. G. Meade. 2016. The CD4(+) T cell methylome contributes to a distinct CD4(+) T cell transcriptional signature in Mycobacterium bovis-infected cattle. Sci Rep 6:31014.

Ettou, S., C. Humbrecht, B. Benet, K. Billot, D. d'Allard, V. Mariot, M. Goodhardt, O. Kosmider, P. Mayeux, E. Solary, and M. Fontenay. 2013. Epigenetic control of NF-kappaB-dependent FAS gene transcription during progression of myelodysplastic syndromes. Mol Cancer Res 11(7):724-735.

Franceschi, C., M. Bonafe, S. Valensin, F. Olivieri, M. De Luca, E. Ottaviani, and G. De Benedictis. 2000. Inflamm-aging. An evolutionary perspective on immunosenescence. Ann N Y Acad Sci 908:244-254.

Glynn, D. J., M. R. Hutchinson, and W. V. Ingman. 2014. Toll-like receptor 4 regulates lipopolysaccharide-induced inflammation and lactation insufficiency in a mouse model of mastitis. Biol Reprod 90(5):91.

Gomes, A. V., S. M. de Souza Morais, S. L. Menezes-Filho, L. G. de Almeida, R. P. Rocha, J. M. Siqueira Ferreira, L. L. Dos Santos, L. C. Malaquias, and L. F. Coelho. 2016. The demethylation profile of the TNF-alpha promoter gene is associated with high expression of this cytokine in Dengue virus patients. J Med Virol.

Gopisetty, G., J. Xu, D. Sampath, H. Colman, and V. K. Puduvalli. 2013. Epigenetic regulation of CD133/PROM1 expression in glioma stem cells by Sp1/myc and promoter methylation. Oncogene 32(26):3119-3129.

Govignon-Gion, A., R. Dassonneville, G. Baloche, and V. Ducrocq. 2015. Multiple trait genetic evaluation of clinical mastitis in three dairy cattle breeds. Animal:1-8.

Gowers, I. R., K. Walters, E. Kiss-Toth, R. C. Read, G. W. Duff, and A. G. Wilson. 2011. Age-related loss of CpG methylation in the tumour necrosis factor promoter. Cytokine 56(3):792-797.

Green, B. B., S. Kandasamy, T. H. Elsasser, and D. E. Kerr. 2011. The use of dermal fibroblasts as a predictive tool of the toll-like receptor 4 response pathway and its development in Holstein heifers. Journal of dairy science 94(11):5502-5514.

Green, B. B. and D. E. Kerr. 2014. Epigenetic contribution to individual variation in response to lipopolysaccharide in bovine dermal fibroblasts. Vet Immunol Immunopathol 157(1-2):49-58.

Green, B. B., S. D. McKay, and D. E. Kerr. 2015. Age dependent changes in the LPS induced transcriptome of bovine dermal fibroblasts occurs without major changes in the methylome. BMC Genomics 16:30.

Gunther, J., W. Petzl, H. Zerbe, H. J. Schuberth, D. Koczan, L. Goetze, and H. M. Seyfert. 2012. Lipopolysaccharide priming enhances expression of effectors of immune defence while decreasing expression of pro-inflammatory cytokines in mammary epithelia cells from cows. BMC Genomics 13:17.

Halim, T. Y., A. MacLaren, M. T. Romanish, M. J. Gold, K. M. McNagny, and F. Takei. 2012. Retinoic-acid-receptor-related orphan nuclear receptor alpha is required for natural helper cell development and allergic inflammation. Immunity 37(3):463-474.

Hawkins, P. T. and L. R. Stephens. 2015. PI3K signalling in inflammation. Biochim Biophys Acta 1851(6):882-897.

Hellwig, S., C. V. Miduturu, S. Kanda, J. Zhang, P. Filippakopoulos, E. Salah, X. Deng, H. G. Choi, W. Zhou, W. Hur, S. Knapp, N. S. Gray, and T. E. Smithgall. 2012. Small-molecule inhibitors of the c-Fes protein-tyrosine kinase. Chem Biol 19(4):529-540.

Huang da, W., B. T. Sherman, and R. A. Lempicki. 2009a. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 37(1):1-13.

Huang da, W., B. T. Sherman, and R. A. Lempicki. 2009b. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature protocols 4(1):44-57.

Kandasamy, S., B. B. Green, A. L. Benjamin, and D. E. Kerr. 2011. Between-cow variation in dermal fibroblast response to lipopolysaccharide reflected in resolution of inflammation during Escherichia coli mastitis. Journal of dairy science 94(12):5963-5975.

Kang, H. S., K. Okamoto, Y. Takeda, J. Y. Beak, K. Gerrish, C. D. Bortner, L. M. DeGraff, T. Wada, W. Xie, and A. M. Jetten. 2011. Transcriptional profiling reveals a role for RORalpha in regulating gene expression in obesity-associated inflammation and hepatic steatosis. Physiol Genomics 43(13):818-828.

Kanzleiter, T., M. Jahnert, G. Schulze, J. Selbig, N. Hallahan, R. W. Schwenk, and A. Schurmann. 2015. Exercise training alters DNA methylation patterns in genes related to muscle growth and differentiation in mice. American journal of physiology. Endocrinology and metabolism 308(10):E912-920.

Keestra-Gounder, A. M., M. X. Byndloss, N. Seyffert, B. M. Young, A. Chavez-Arroyo, A. Y. Tsai, S. A. Cevallos, M. G. Winter, O. H. Pham, C. R. Tiffany, M. F. de Jong, T. Kerrinnes, R. Ravindran, P. A. Luciw, S. J. McSorley, A. J. Baumler, and R. M. Tsolis. 2016. NOD1 and NOD2 signalling links ER stress with inflammation. Nature 532(7599):394-397.

Koga, Y., T. Hisada, T. Ishizuka, M. Utsugi, A. Ono, M. Yatomi, Y. Kamide, H. Aoki-Saito, H. Tsurumaki, K. Dobashi, and M. Yamada. 2016. CREB regulates TNF-alpha-induced GM-CSF secretion via p38 MAPK in human lung fibroblasts. Allergol Int 65(4):406-413.

Kok, B. P. and E. Saez. 2014. Activating PI3-kinase to dampen inflammation. Chem Biol 21(8):917-918.

Kornalijnslijper, E., B. Beerda, I. Daemen, J. van der Werf, T. van Werven, T. Niewold, V. Rutten, and E. Noordhuizen-Stassen. 2003. The effect of milk production level on host resistance of dairy cows, as assessed by the severity of experimental Escherichia coli mastitis. Vet Res 34(6):721-736.

Labzin, L. I., S. V. Schmidt, S. L. Masters, M. Beyer, W. Krebs, K. Klee, R. Stahl, D. Lutjohann, J. L. Schultze, E. Latz, and D. De Nardo. 2015. ATF3 Is a Key Regulator of Macrophage IFN Responses. J Immunol 195(9):4446-4455.

Lacerte, P., A. Brunet, B. Egarnes, B. Duchene, J. P. Brown, and J. Gosselin. 2016. Overexpression of TLR2 and TLR9 on monocyte subsets of active rheumatoid arthritis patients contributes to enhance responsiveness to TLR agonists. Arthritis Res Ther 18(1):10.

Lee, G. L., J. Y. Wu, C. C. Yeh, and C. C. Kuo. 2016. TLR4 induces CREB-mediated IL-6 production via upregulation of F-spondin to promote vascular smooth muscle cell migration. Biochem Biophys Res Commun 473(4):1205-1210.

Lee, S. M., E. J. Kim, K. Suk, and W. H. Lee. 2011. BAFF and APRIL induce inflammatory activation of THP-1 cells through interaction with their conventional receptors and activation of MAPK and NF-kappaB. Inflamm Res 60(9):807-815.
Lemaitre, B., E. Nicolas, L. Michaut, J. M. Reichhart, and J. A. Hoffmann. 1996. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell 86(6):973-983.

Lents, C. A., R. P. Wettemann, M. J. Paape, J. A. Vizcarra, M. L. Looper, D. S. Buchanan, and K. S. Lusby. 2002. Efficacy of intramuscular treatment of beef cows with

oxytetracycline to reduce mastitis and to increase calf growth. Journal of animal science 80(6):1405-1412.

Li, J., R. Li, Y. Wang, X. Hu, Y. Zhao, L. Li, C. Feng, X. Gu, F. Liang, S. J. Lamont, S. Hu, H. Zhou, and N. Li. 2015. Genome-wide DNA methylome variation in two genetically distinct chicken lines using MethylC-seq. BMC Genomics 16:851.

Marttila, S., L. Kananen, S. Hayrynen, J. Jylhava, T. Nevalainen, A. Hervonen, M. Jylha, M. Nykter, and M. Hurme. 2015. Ageing-associated changes in the human DNA methylome: genomic locations and effects on gene expression. BMC Genomics 16:179.

Michel, T., J. M. Reichhart, J. A. Hoffmann, and J. Royet. 2001. Drosophila Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. Nature 414(6865):756-759.

Mohammed-Ali, Z., G. L. Cruz, and J. G. Dickhout. 2015. Crosstalk between the unfolded protein response and NF-kappaB-mediated inflammation in the progression of chronic kidney disease. J Immunol Res 2015:428508.

Nile, C. J., R. C. Read, M. Akil, G. W. Duff, and A. G. Wilson. 2008. Methylation status of a single CpG site in the IL6 promoter is related to IL6 messenger RNA levels and rheumatoid arthritis. Arthritis Rheum 58(9):2686-2693.

Pan, H., L. Chen, S. Dogra, A. L. Teh, J. H. Tan, Y. I. Lim, Y. C. Lim, S. Jin, Y. K. Lee, P. Y. Ng, M. L. Ong, S. Barton, Y. S. Chong, M. J. Meaney, P. D. Gluckman, W. Stunkel, C. Ding, and J. D. Holbrook. 2012. Measuring the methylome in clinical samples: improved processing of the Infinium Human Methylation450 BeadChip Array. Epigenetics 7(10):1173-1187.

Pareek, R., O. Wellnitz, R. Van Dorp, J. Burton, and D. Kerr. 2005. Immunorelevant gene expression in LPS-challenged bovine mammary epithelial cells. J Appl Genet 46(2):171-177.

Pena, C. J., Y. D. Neugut, and F. A. Champagne. 2013. Developmental timing of the effects of maternal care on gene expression and epigenetic regulation of hormone receptor levels in female rats. Endocrinology 154(11):4340-4351.

Persson Waller, K., Y. Persson, A. K. Nyman, and L. Stengarde. 2014. Udder health in beef cows and its association with calf growth. Acta Vet Scand 56:9.

Petzl, W., J. Gunther, T. Pfister, C. Sauter-Louis, L. Goetze, S. von Aulock, A. Hafner-Marx, H. J. Schuberth, H. M. Seyfert, and H. Zerbe. 2012. Lipopolysaccharide pretreatment of the udder protects against experimental Escherichia coli mastitis. Innate Immun 18(3):467-477.

Rudolf, R., R. Busch, A. K. Patra, K. Muhammad, A. Avots, J. C. Andrau, S. Klein-Hessling, and E. Serfling. 2014. Architecture and expression of the nfatc1 gene in lymphocytes. Front Immunol 5:21.

Sacramento, L., S. C. Trevelin, M. S. Nascimento, D. S. Lima-Junior, D. L. Costa, R. P. Almeida, F. Q. Cunha, J. S. Silva, and V. Carregaro. 2015. Toll-like receptor 9 signaling in dendritic cells regulates neutrophil recruitment to inflammatory foci following Leishmania infantum infection. Infect Immun 83(12):4604-4616.

Salazar-Camarena, D. C., P. C. Ortiz-Lazareno, A. Cruz, E. Oregon-Romero, J. R. Machado-Contreras, J. F. Munoz-Valle, M. Orozco-Lopez, M. Marin-Rosales, and C. A. Palafox-Sanchez. 2016. Association of BAFF, APRIL serum levels, BAFF-R, TACI and BCMA expression on peripheral B-cell subsets with clinical manifestations in systemic lupus erythematosus. Lupus 25(6):582-592.

Sandberg, W. J., K. Otterdal, L. Gullestad, B. Halvorsen, A. Ragnarsson, S. S. Froland, J. K. Damas, E. Oie, P. Aukrust, G. K. Hansson, and A. Yndestad. 2009. The tumour necrosis factor superfamily ligand APRIL (TNFSF13) is released upon platelet activation and expressed in atherosclerosis. Thromb Haemost 102(4):704-710.

Sharma, B. S., M. K. Abo-Ismail, F. S. Schenkel, Q. You, C. P. Verschoor, S. D. Pant, and N. A. Karrow. 2015. Association of TLR4 polymorphisms with Mycobacterium avium subspecies paratuberculosis infection status in Canadian Holsteins. Anim Genet 46(5):560-565.

Smith, J. A., L. A. Samayawardhena, and A. W. Craig. 2010. Fps/Fes protein-tyrosine kinase regulates mast cell adhesion and migration downstream of Kit and beta1 integrin receptors. Cell Signal 22(3):427-436.

Starkhammar, M., S. Kumlien Georen, S. E. Dahlen, L. O. Cardell, and M. Adner. 2015. TNFalpha-blockade stabilizes local airway hyperresponsiveness during TLR-induced exacerbations in murine model of asthma. Respir Res 16:129.

Stifter, S. A. and C. G. Feng. 2015. Interfering with immunity: detrimental role of type I IFNs during infection. J Immunol 194(6):2455-2465.

Stringhini, S., S. Polidoro, C. Sacerdote, R. S. Kelly, K. van Veldhoven, C. Agnoli, S. Grioni, R. Tumino, M. C. Giurdanella, S. Panico, A. Mattiello, D. Palli, G. Masala, V. Gallo, R. Castagne, F. Paccaud, G. Campanella, M. Chadeau-Hyam, and P. Vineis. 2015. Life-course socioeconomic status and DNA methylation of genes regulating inflammation. Int J Epidemiol 44(4):1320-1330.

Stringhini, S., P. Zaninotto, M. Kumari, M. Kivimaki, and G. D. Batty. 2016. Lifecourse socioeconomic status and type 2 diabetes: the role of chronic inflammation in the English Longitudinal Study of Ageing. Sci Rep 6:24780.

Sturdevant, G. L. and H. D. Caldwell. 2014. Innate immunity is sufficient for the clearance of Chlamydia trachomatis from the female mouse genital tract. Pathog Dis 72(1):70-73.
Suehiro, J., T. Hamakubo, T. Kodama, W. C. Aird, and T. Minami. 2010. Vascular endothelial growth factor activation of endothelial cells is mediated by early growth response-3. Blood 115(12):2520-2532.

Takahashi, K., Y. Sugi, A. Hosono, and S. Kaminogawa. 2009. Epigenetic regulation of TLR4 gene expression in intestinal epithelial cells for the maintenance of intestinal homeostasis. J Immunol 183(10):6522-6529.

Tan, B. L., M. N. Yazicioglu, D. Ingram, J. McCarthy, J. Borneo, D. A. Williams, and R. Kapur. 2003. Genetic evidence for convergence of c-Kit- and alpha4 integrin-mediated signals on class IA PI-3kinase and the Rac pathway in regulating integrin-directed migration in mast cells. Blood 101(12):4725-4732.

Thakur, B. K., N. Dasgupta, A. Ta, and S. Das. 2016. Physiological TLR5 expression in the intestine is regulated by differential DNA binding of Sp1/Sp3 through simultaneous Sp1 dephosphorylation and Sp3 phosphorylation by two different PKC isoforms. Nucleic Acids Res 44(12):5658-5672.

Uchiyama, R., B. Chassaing, B. Zhang, and A. T. Gewirtz. 2015. MyD88-mediated TLR signaling protects against acute rotavirus infection while inflammasome cytokines direct Ab response. Innate Immun 21(4):416-428.

van Delft, M. A., L. F. Huitema, and S. W. Tas. 2015. The contribution of NF-kappaB signalling to immune regulation and tolerance. Eur J Clin Invest 45(5):529-539.

van Eijk, K. R., S. de Jong, M. P. Boks, T. Langeveld, F. Colas, J. H. Veldink, C. G. de Kovel, E. Janson, E. Strengman, P. Langfelder, R. S. Kahn, L. H. van den Berg, S. Horvath, and R. A. Ophoff. 2012. Genetic analysis of DNA methylation and gene expression levels in whole blood of healthy human subjects. BMC Genomics 13:636.

Vu, T. H., T. Li, and A. R. Hoffman. 2004. Promoter-restricted histone code, not the differentially methylated DNA regions or antisense transcripts, marks the imprinting status of IGF2R in human and mouse. Hum Mol Genet 13(19):2233-2245.

Wang, B., Y. H. Rao, M. Inoue, R. Hao, C. H. Lai, D. Chen, S. L. McDonald, M. C. Choi, Q. Wang, M. L. Shinohara, and T. P. Yao. 2014. Microtubule acetylation amplifies p38 kinase signalling and anti-inflammatory IL-10 production. Nat Commun 5:3479.

Weldon, A. J., I. Moldovan, M. G. Cabling, E. A. Hernandez, S. Hsu, J. Gonzalez, A. Parra, A. Benitez, N. Daoud, K. Colburn, and K. J. Payne. 2015. Surface APRIL Is Elevated on Myeloid Cells and Is Associated with Disease Activity in Patients with Rheumatoid Arthritis. J Rheumatol 42(5):749-759.

Wen, A. Y., K. M. Sakamoto, and L. S. Miller. 2010. The role of the transcription factor CREB in immune function. J Immunol 185(11):6413-6419.
Williams, D. L., C. F. Li, T. Z. Ha, T. Ozment-Skelton, J. H. Kalbfleisch, J. Preiszner, L. Brooks, K. Breuel, and J. B. Schweitzer. 2004. Modulation of the phosphoinositide innate resistance to polymicrobial 3-kinase pathway alters sepsis. Journal of Immunology 172(1):449-456.

Yarilina, A., K. Xu, J. Chen, and L. B. Ivashkiv. 2011. TNF activates calcium-nuclear factor of activated T cells (NFAT)c1 signaling pathways in human macrophages. Proc Natl Acad Sci U S A 108(4):1573-1578.

Zanoni, I., R. Ostuni, G. Capuano, M. Collini, M. Caccia, A. E. Ronchi, M. Rocchetti, F. Mingozzi, M. Foti, G. Chirico, B. Costa, A. Zaza, P. Ricciardi-Castagnoli, and F. Granucci. 2009. CD14 regulates the dendritic cell life cycle after LPS exposure through NFAT activation. Nature 460(7252):264-268.

Zhang, X., R. Yang, Y. Jia, D. Cai, B. Zhou, X. Qu, H. Han, L. Xu, L. Wang, Y. Yao, and G. Yang. 2014. Hypermethylation of Sp1 binding site suppresses hypothalamic POMC in neonates and may contribute to metabolic disorders in adults: impact of maternal dietary CLAs. Diabetes 63(5):1475-1487.

**Table 2.1: Real Time PCR Primers.**

| Gene Name | Sequence 5' to 3' | Reference |
|---|---|---|
| β-Actin | F: GCAAATGCTTCTAGGCGGACT<br>R: CAATCTCATCTCGTTTTCTGCG | (Pareek et al., 2005) |
| Feline Sarcoma Oncogene (FES) | F: GTCTCAGACAAGTCCCCGTG<br>R: AGTCTGAACACAGCGTCAGG | Designed in house |
| Interleukin 6 (IL6) | F: TGAGGGAAATCAGGAAAATGT<br>R: CAGTGTTTGTGGCTGGAGTG | (Pareek et al., 2005) |
| Interleukin 8 (IL8) | F: GCTGGCTGTTGCTCTCTTG<br>R: AGGTGTGGAATGTGTTTTTATGC | (Pareek et al., 2005) |
| Nuclear factor of activated T-cells calcineurin dependent 1 (NFATc1) | F: GTCCGACGTCAAGCGGTAG<br>R: TTGACCGTTACGGGAATGGG | Designed in house |
| Phosphatidylinositol 3-kinase regulatory subunit alpha (PIK3R1) | F: TCATTCCGGTAGCCGTTTCC<br>R: CTCAGAACTTGCTGCTGGGA | Designed in house |
| RAR-Related Orphan Receptor A (RORA) | F: ATAACATCTCGGCCAACGGG<br>R: GGAAGAAGCCTGATGCTGGT | Designed in house |
| Transcription factor 7 (T-cell specific, HMG-box) (TCF7) | F: GAGCCAAAGTCATTGCGGAG<br>R: TCTTTTTCCTCCTGAGTTGGATTC | Designed in house |
| Tumor Necrosis Factor Super Family 13 (TNFSF13) | F: AGAAGCGCTCAGTTCTGCAT<br>R: CTGTTGTAGGCCCAGTCAGG | Designed in house |

**Table 2.1: Suggestive Transcription Factor Enrichment of Gene Promoters More Methylated in Young Cultures.**

| Category | Term | Count* | Fold Enrichment** | FDR*** |
|---|---|---|---|---|
| UCSC_TFBS | PAX5 | 288 | 1.32 | 4.35E-09 |
| UCSC_TFBS | LMO2COM | 297 | 1.31 | 6.04E-09 |
| UCSC_TFBS | P53 | 321 | 1.25 | 2.23E-08 |
| UCSC_TFBS | EGR3 | 111 | 1.77 | 5.34E-08 |
| UCSC_TFBS | AP2 | 148 | 1.57 | 1.04E-07 |
| UCSC_TFBS | NMYC | 174 | 1.48 | 1.57E-07 |
| UCSC_TFBS | ARNT | 243 | 1.33 | 2.26E-07 |
| UCSC_TFBS | NRSF | 265 | 1.29 | 3.42E-07 |
| UCSC_TFBS | MYCMAX | 302 | 1.23 | 8.19E-07 |
| UCSC_TFBS | NFKB | 246 | 1.30 | 1.43E-06 |
| UCSC_TFBS | AHRARNT | 259 | 1.28 | 1.49E-06 |
| UCSC_TFBS | CREB | 218 | 1.33 | 1.79E-06 |
| UCSC_TFBS | E47 | 290 | 1.23 | 2.99E-06 |
| UCSC_TFBS | COUP | 209 | 1.33 | 5.73E-06 |
| UCSC_TFBS | TAXCREB | 262 | 1.25 | 6.31E-06 |

*Number of inputted genes regulated by transcription factor listed.
**Fold enrichment as measured by Fisher's Exact Test.
***Benjamini-Hochberg False Discovery Rate.

**Table 2.2: Suggestive Transcription Factor Enrichment of Gene Promoters More Methylated in Old Cultures.**

| Category | Term | Count* | Fold Enrichment** | FDR*** |
|---|---|---|---|---|
| UCSC_TFBS | PAX5 | 187 | 1.43 | 8.40E-10 |
| UCSC_TFBS | SP1 | 95 | 1.73 | 1.66E-06 |
| UCSC_TFBS | ATF | 113 | 1.58 | 3.02E-06 |
| UCSC_TFBS | NRSF | 165 | 1.35 | 9.59E-06 |
| UCSC_TFBS | MIF1 | 136 | 1.43 | 1.06E-05 |
| UCSC_TFBS | P300 | 124 | 1.47 | 1.09E-05 |
| UCSC_TFBS | ZID | 150 | 1.38 | 1.14E-05 |
| UCSC_TFBS | SREBP1 | 178 | 1.28 | 4.10E-05 |
| UCSC_TFBS | MYOGNF1 | 145 | 1.35 | 6.27E-05 |
| UCSC_TFBS | TAXCREB | 162 | 1.30 | 8.37E-05 |
| UCSC_TFBS | ZIC3 | 123 | 1.41 | 8.80E-05 |
| UCSC_TFBS | MZF1 | 147 | 1.33 | 9.46E-05 |
| UCSC_TFBS | AHRARNT | 158 | 1.31 | 9.57E-05 |
| UCSC_TFBS | HMX1 | 139 | 1.34 | 1.39E-04 |
| UCSC_TFBS | TAL1BETAITF2 | 158 | 1.29 | 1.50E-04 |

*Number of inputted genes regulated by transcription factor listed.
**Fold enrichment as measured by Fisher's Exact Test.
***Benjamini-Hochberg False Discovery Rate.

**Table 2.3: Conserved Transcription Factor Enrichment of Gene Promoters More Methylated in Young Cultures.**

| Category | Term | Count* | Fold Enrichment** | FDR*** |
|---|---|---|---|---|
| UCSC_TFBS | P300 | 49 | 1.67 | 0.007 |
| UCSC_TFBS | TAL1BETAITF2 | 60 | 1.40 | 0.024 |
| UCSC_TFBS | CDC5 | 52 | 1.46 | 0.027 |
| UCSC_TFBS | ROAZ | 52 | 1.44 | 0.027 |
| UCSC_TFBS | P53 | 69 | 1.29 | 0.028 |
| UCSC_TFBS | COUP | 48 | 1.46 | 0.031 |
| UCSC_TFBS | NFKB | 56 | 1.42 | 0.032 |
| UCSC_TFBS | EGR3 | 25 | 1.92 | 0.032 |
| UCSC_TFBS | AP2 | 35 | 1.78 | 0.032 |
| UCSC_TFBS | AP2GAMMA | 18 | 2.19 | 0.040 |

*Number of inputted genes regulated by transcription factor listed.
**Fold enrichment as measured by Fisher's Exact Test.
***Benjamini-Hochberg False Discovery Rate.

**Table 2.4: Summary of Selected Innate Response Genes Hyper-Methylated in Young Cultures.**

| Gene Name | Number of Differentially Methylated Sites* | Location of Differentially Methylated Sites | Expression (Old>Young) at 0, 2 & 8 H post LPS** | Expression (Holstein>Angus) at 0, 2, & 8 H post LPS** |
|---|---|---|---|---|
| FES | 2 | Promoter, Exon | 5.7, 4.3, 6.3 | 3.7, 5.4, 4.8 |
| NFATc1 | 3 | Promoter, Intron | 1.8, 1.8, --- | 2.2, 6.5, 1.7 |
| PIK3R1 | 2 | Promoter, Intron | 3.0, 2.6, 2.5 | ---, 2.4, (-1.9) |
| RORA | 5 | Intron | ---, ---, 2.1 | 2.0, 2.2, --- |
| TNFSF13 | 1 | Promoter | 2.0, 1.6, 2.8 | ---, ---, --- |
| TCF7 | 2 | Promoter | ---, ---, --- | 4.5, ---, 4.3 |

*Number of differentially methylated CpGs (P<0.05, >25% meth diff).
**Displayed as fold change at 0, 2 or 8 H post LPS. Positive values indicate (Old > Young) or (Holstein >Angus), negative values indicate (Young > Old) or (Angus > Holstein) and --- signifies P>0.10.

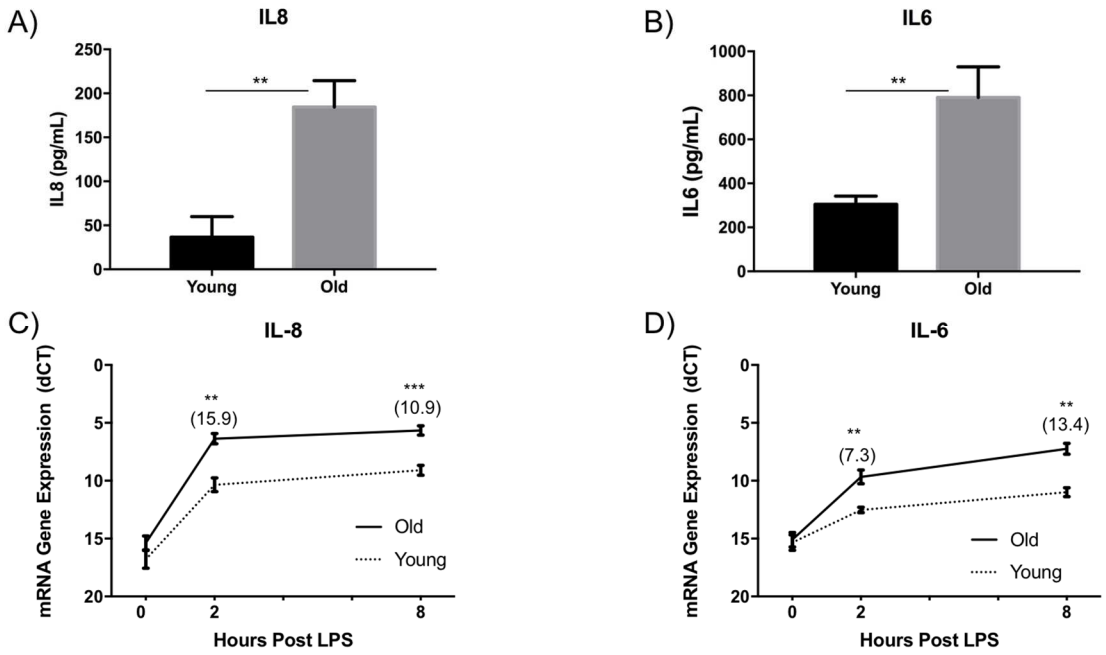


**Figure 2.1: Fibroblast Response to LPS in Young versus Old Cultures.**

Interleukin-8 (A & C) and Interleukin-6 (B & D) protein production and gene expression were measured in young and old dermal fibroblasts (n = 6 per group) at various time points post LPS. Protein production is presented in pg/ml following 36 hours of LPS stimulation. Gene expression was measured by RT-qPCR and is presented as the change in cycles to threshold (dCT) of gene expression at 0, 2 & 8 hours post LPS in comparison to β-actin. Fold change gene expression (Old>Young) is presented in parentheses above each significant time point. All values are displayed as mean (+/- SEM). Significance was measured using a paired Student's t test at each time point and ** = $P < 0.01$ *** = $P < 0.001$.

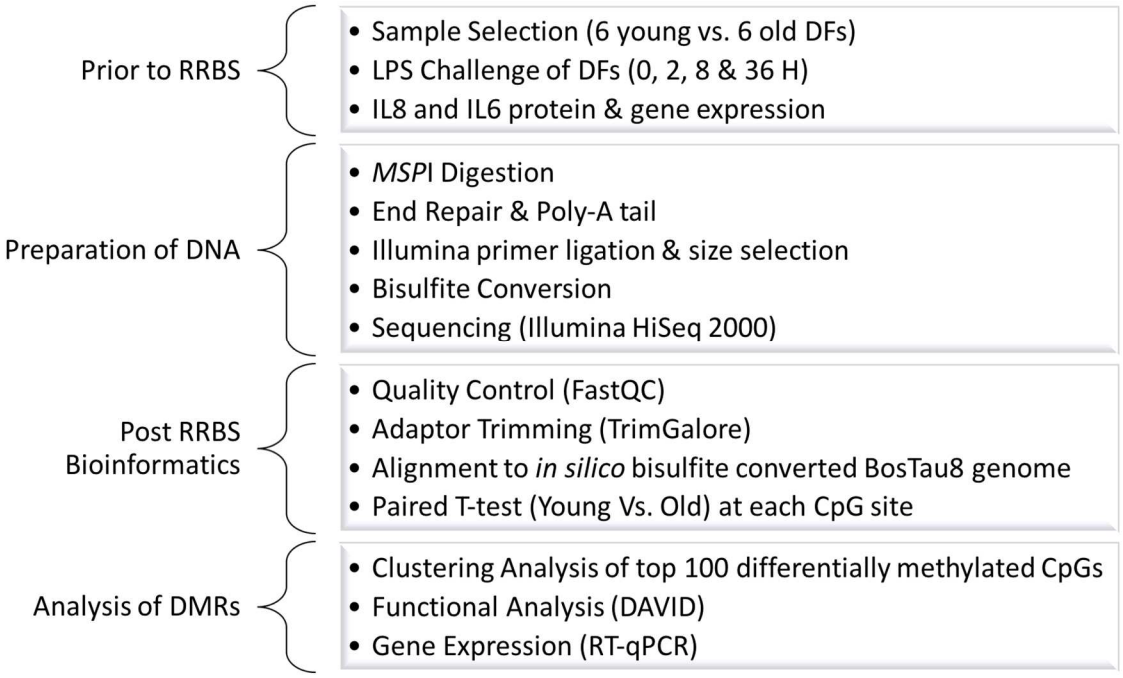


**Figure 2.2: RRBS Project Workflow**

Prior to methylation analysis, 6 pairs of fibroblasts isolated from the same animals at 5- and 16-months of age were selected and challenged with LPS for 0, 2, 8 and 36 hours to measure cytokine protein and gene expression. In preparation for RRBS, fibroblast DNA was digested with MSPI to enrich for CpG rich regions of the genome, followed by end-repair and Poly-A tail addition. The digested DNA was then ligated to Illumina primers, size selected and bisulfite converted prior to sequencing. The subsequent reads were quality controlled by FastQC and Illumina adaptors were trimmed with TrimGalore. Those that passed quality control were then aligned to the BosTau8 genome and uniquely mapped reads were kept for analysis with a paired T test at each CpG site with greater than or equal to 5X coverage. Once differentially methylated sites were identified, clustering analysis was performed on the top 100 sites based on p-value. Functional analysis to identify enriched KEGG pathways, GO terms and transcription factors was performed by DAVID and gene expression analysis by RT-qPCR.

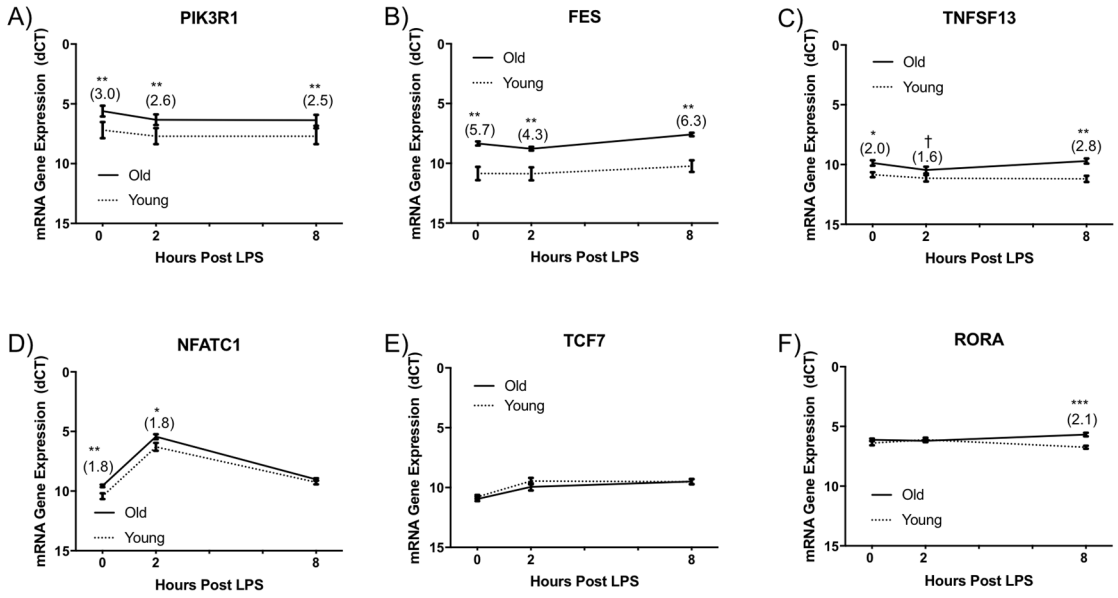


**Figure 2.3: Gene Expression post LPS treatment of Selected Differentially Methylated Genes in Young and Old Fibroblast Cultures.**

Expression of six genes more methylated in young cultures A) PIK3R1, B) FES, C) TNFSF13, D) NFATC1, E) TCF7 and F) RORA were measured in young and old fibroblasts (n = 6 per group) at 0, 2 and 8 hours post LPS. Expression was measured by RT-qPCR and values are presented as dCT in comparison to β-actin. Fold change gene expression (Old>Young) is displayed in parentheses above each significantly different time points. All values are displayed as mean (+/- SEM). Significance was determined using a paired Student's t Test at each time point post LPS treatment and *** = P < 0.001, ** = P<0.01, * = P < 0.05, † = P < 0.10.

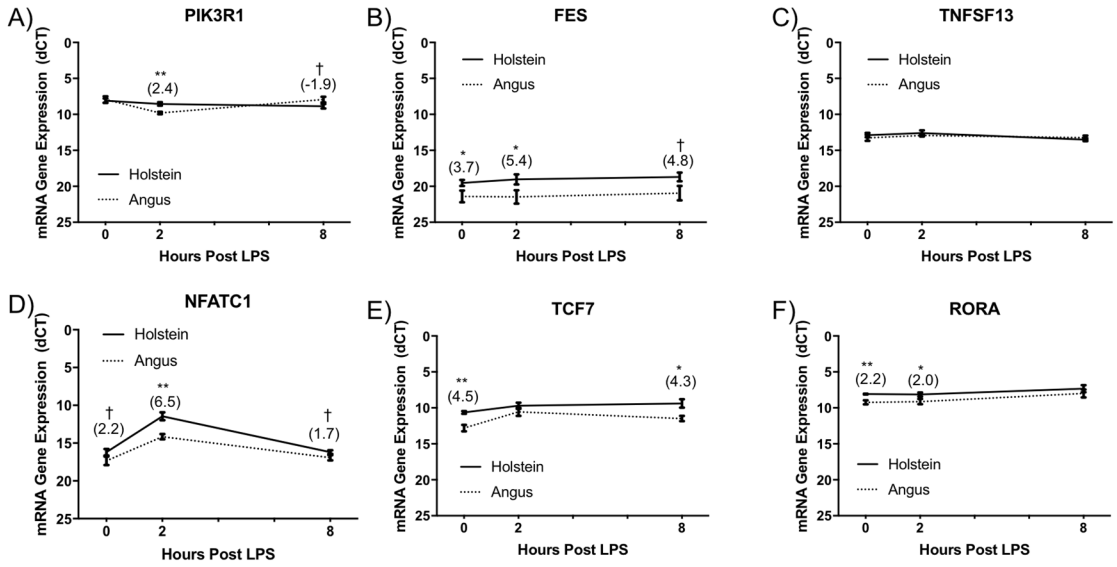


**Figure 2.4: Gene Expression post LPS Treatment of Selected Genes from RRBS Analysis in Angus and Holstein Fibroblast Cultures.**

Expression of six genes more methylated in young cultures, A) PIK3R1, B) FES, C) TNFSF13, D) NFATC1, E) TCF7 and F) RORA were measured in fibroblasts isolated from 19-month Angus and Holstein cows at 0, 2 and 8 hours post LPS. Expression was measured by RT-qPCR and values are presented as dCT in comparison to β-actin. Fold change gene expression is displayed in parentheses above each significantly different time point, whereby positive values indicate Holstein>Angus and negative values indicate Angus>Holstein. All values are displayed as mean (+/- SEM). Significance was determined using an unpaired Student's t Test at each time point post LPS treatment and ** = P<0.01, * = P < 0.05, † = P < 0.10.

Additional files for Chapter 2 can be accessed on NCBI's gene expression omnibus (GEO) website through GEO accession number GSE91088.

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE91088

# CHAPTER 3: VARIATION IN FIBROBLAST EXPRESSION OF TLR4 AND LPS-INDUCED CYTOKINE PRODUCTION BETWEEN-ANIMALS PREDICTS CONTROL OF BACTERIAL GROWTH BUT NOT SEVERITY OF *ESCHERICHIA COLI* MASTITIS

## 3.1 Abstract

Mastitis cases caused by environmental pathogens, such as *Escherichia coli* (*E. coli*) are highly problematic to the dairy industry because milk producers incur substantial cost and the disease tends to be difficult to manage. An effective innate immune response by the host is key to controlling infection, but it should also limit collateral damage to the mammary gland. Between-animal differences in mastitis severity have been attributed to variability in the innate response. In the current study, the primary dermal fibroblast was used as a model to rank animals based on composite expression of toll-like receptor 4 (*TLR4*) and lipopolysaccharide (LPS)-induced IL-8 and IL-6 protein production. Animals ranked as high and low were then infected in the mammary gland with the P4 strain of *E. coli* to determine how difference in rank would affect response to mastitis. All animals developed an acute response to the infection with varying degrees in severity, however, high responders had an elevated somatic cell count and fever response at 12-hrs. post infection and higher quantities of milk IL-8 at 24-hrs. post infection. High responding animals were also significantly more capable of limiting bacterial growth. No differences were measured in post-infection milk production or tissue damage. The current study indicates that high responding animals have an early up-regulation in their innate response that is beneficial for bacterial clearance, however they are still equally susceptible to tissue damage caused by an exuberant response to the infection. The

dermal fibroblast can be used in conjunction with other cell types to determine how the innate response is regulated to mitigate unnecessary injury to the mammary gland while still effectively clearing the pathogen.

Keywords: LPS; experimental mastitis; dairy cow; innate immunity

## 3.2 Introduction

Infection of the mammary gland with *Escherichia coli* (*E. coli*), a common opportunistic pathogen found within the dairy cow environment, causes clinical mastitis that ranges from mild to severe. Although infections are typically transient and self-limiting, host response to the pathogen causes damage to milk-producing epithelial cells that results in a substantial decrease in milk production and quality. In addition to lower milk production and non-saleable milk, direct costs, such as veterinary care, and indirect costs, such as early culling, contribute to mastitis-related economic losses that altogether cost the producer approximately $450 per clinical case of mastitis occurring within the first 30 days of lactation (Rollin et al., 2015). Treatment of *E. coli* mastitis with antibiotics has shown very limited benefit (Lago et al., 2011b, a, Vasquez et al., 2017) and, within the United States, effective treatments are restricted to hetacillin potassium, a broad-spectrum ß-lactam antibiotic, and ceftiofur, a third generation cephalosporin and a high priority drug for human diseases (Suojala et al., 2013); thus their use in animal agriculture is not generally recommended. Use of the current J5 vaccine against *E. coli* has shown a modest benefit in reducing severity of mastitis (Wilson et al., 2007), however, it does not significantly reduce the incidence of disease. Both the desire to reduce antibiotic usage and a lack of an efficacious vaccine has led to increased interest

in the selection of animals based on natural disease resistance, however, genes that contribute to resistance and susceptibility have remained relatively elusive.

Physiological state of the host is known to affect mastitis severity and susceptibility, whereby early lactation, older age and increased parity are risk factors for mastitis (Steeneveld et al., 2008, Taponen et al., 2017). Differences in strain of *E. coli* may also contribute to variation in mastitis severity (Blum et al., 2017). It has been suggested that strains adapted to the mammary gland, called mammary pathogenic strains (MPEC), express virulence factors and more efficiently utilize nutrient sources that allow for improved survival within the mammary gland environment (Blum et al., 2015). However, a recent analysis comparing the gene sequences of eight MPEC strains versus six fecal commensal strains did not reveal enrichment of virulence determinants or fitness factors in strains isolated from bovine mastitis cases versus commensal organisms. Instead, the authors suggest that each individual strain contains unique strain-specific traits that enhance fitness and are the result of competition for resources associated with commensalism rather than pathogenicity (Leimbach et al., 2017). In addition, experimental infections controlling for host variables, such as stage of lactation and parity, and with the same strain of *E. coli,* have shown that the severity of clinical mastitis is quite variable between animals (Lee et al., 2006), albeit to a lesser extent in primiparous cows (Vangroenweghe et al., 2004, Vangroenweghe et al., 2005a). Additional host factors, such as the genetic or epigenetic regulation of immunity, may help to explain variation in mastitis severity between animals that is independent of bacteria and other host factors.

Genetic predisposition to infectious disease, including mastitis, has been widely studied, but is complicated by the likely polygenic nature of this trait. In one study, using the estimated breeding values (EBV) of bulls based on daughter somatic cell score (SCS) as the primary risk factor for mastitis susceptibility, genetic polymorphisms in the gene region of osteopontin (*SPP1*) were shown to have an effect on SCS and presumably mastitis risk (Alain et al., 2009). In addition, a quantitative trait loci (QTL) on chromosome 18 is associated with resistance or susceptibility to mastitis. Interestingly, mammary epithelial cells that are isolated from animals with either the resistant or susceptible QTL also exhibit differences in the up-regulation and kinetics of numerous innate immune response genes in response to *E. coli* and *S. aureus* in vitro stimulation (Brand et al., 2011). Furthermore, genes that exhibit differences in DNA methylation have been identified in peripheral blood leukocytes isolated from cows with subclinical *S. aureus* mastitis versus uninfected cattle, and include three inflammatory response genes that are hypo-methylated and up-regulated in infected cattle (Song et al., 2016). The three aforementioned studies, among many others, emphasize the importance of the innate immune response to mastitis and seem to suggest that differences in innate immunity may determine mastitis resistance or susceptibility.

In response to infection, the innate immune response is rapidly activated through the interaction of germ-line encoded pattern recognition receptors (PRR) with conserved microbial components. Toll-like Receptor 4 (TLR4) is the main PRR that is activated in response to Gram-negative bacteria, such as *E. coli*, as a result of its interaction with bacterial endotoxin, or LPS (Chow et al., 1999). Following recognition of LPS facilitated by co-receptors CD14 and MD2, TLR4 activates both a MyD88-dependent and -

independent signaling cascade that allows transcription factors NF-κB, AP-1 and IRFs to up-regulate the transcription of inflammatory response genes, such as *IL-8, IL-6, TNF-α, IL-1β*, and *IFN-γ*, among many others (Lu et al., 2008). To determine how innate immunity contributes to between-animal differences in response to mastitis-causing pathogens, we have developed a primary bovine dermal fibroblast model to assess the immune response of a large number of animals in cell culture prior to testing the in vivo response of selected animals to intra-mammary infection. The dermal fibroblast was chosen as a model cell for its repeatability of response (Kandasamy et al., 2011), ease of isolation and cryopreservation, and low activation at baseline. Furthermore, a comparison of model cell types including primary bovine epithelial cells, fibroblasts and macrophages showed that while both fibroblasts and epithelial cells distinguished between pathogen-specific immune responses, macrophages fail to make the distinction (Gunther et al., 2016b).

Using this approach, we have previously ranked cohorts of adult Holsteins on fibroblast IL-8 production in response to LPS or PAM2CSK4 to mimic *E. coli* and *S. aureus* mastitis, respectively. Animals that were designated as high responders and low responders, based on high or low fibroblast IL-8 production, were then infected with the pathogen of interest. Both experiments showed that high responders had elevated tissue damage and somatic cell count within the mammary gland in response to infection. High responders challenged with *E. coli* also had a slower return to pre-infection milk production levels, and high responders challenged with *S. aureus* had significantly elevated milk IL-8. In either case, no difference was measured in bacterial clearance, suggesting that high responders do not benefit from the enhanced response and that the

innate response contributes to mammary gland tissue damage and lower milk production (Kandasamy et al., 2011, Benjamin et al., 2015b).

To further characterize the high response phenotype and potentially identify genetic markers for severe mastitis susceptibility, the current experiment utilizes a multi-variable approach to rank a cohort of adult Holsteins on LPS-induced fibroblast production of IL-8 and IL-6 and gene expression of *TLR4*. High and low responders were subsequently infected with *E. coli* to determine if fibroblast phenotype would accurately reflect the immune response within the mammary gland.

## 3.3 Materials and Methods

### 3.3.1 Ethics Statement

All animal procedures performed in this experiment were approved by the Institutional Animal Care and Use Committee at the University of Vermont. Animals enrolled in the study were handled by authorized personnel approved by the aforementioned committee and all efforts were made to minimize animal suffering in accordance with IACUC standards. In particular, Banamine® was administered to animals that developed a febrile response of greater than 105.7 ºF (40.9ºC).

### 3.3.2 Experimental Animals

Adult Holstein cows were housed at a large (1,400 lactating cows), local collaborating dairy farm and 60 early lactation animals were selected to be included in the study. At the time of the initial skin sampling, all animals were in early lactation (78 ± 10 DIM) and were in their second or greater (2.9 ± 1.1) lactation. The 12 animals selected for the intra-mammary treatment with *E. coli* were transported to the University of Vermont's Miller Research farm when they were in late lactation (238 ± 6 DIM). They

were housed in a tie-stall barn, fed a standard total-mixed ration *ad libitum,* and allowed a seven-day acclimation period prior to challenge. Selected cows were in their $2^{nd}$ – $4^{th}$ lactation.

### 3.3.3 Dermal Fibroblast Isolation and Culturing

Ear notches from 60 animals were collected over a 4-week period with 15 animals sampled per week. The section of the ear to be sampled was clipped and shaved to remove hair and scrubbed three times, alternating between povidone iodine and 70% ethanol. After the last ethanol wash, a large v-cut ear notch was taken using an aluminum ear notcher (Model# WI-0002433, Livestock Concepts, Hawarden, IA), while carefully avoiding main arteries and vein. Ear notch samples were transported to the laboratory on ice in Dulbecco's PBS (DPBS; Hyclone Laboratories, Logan, UT) containing a 1x antibiotic-antimycotic solution (1x AB/AM; 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 ug/ml of amphotericin B; Hyclone Laboratories). Tissue samples were then minced into smaller pieces using opposing scalpel blades and washed 4 times with fresh DPBS containing 1x AB/AM. After the last wash, 10 ml of Dulbecco's modified Eagle medium (DMEM; Hyclone) containing 1x AB/AM and 0.5% collagenase type I enzyme (Gibco, Gaithersburg, MD) was added to each sample and samples were incubated overnight at 37°C with orbital shaking. The collagenase-digested tissue was then filtered with a 70 µM nylon mesh filter (Thermo Fisher, Pittsburgh, PA) and centrifuged at 1100 x *g* for 5 minutes at room temperature. Supernatant was discarded and the cell pellet was reconstituted in DMEM containing 10% fetal bovine serum (FBS; Omega Scientific, Tarzana, CA), 1x insulin-transferrin-selenium (ITS; Gibco), and 1x AB/AM. Cells were cultured in a 25 $cm^2$ flask in a humidified incubator containing 5% $CO_2$ at 37°C until

reaching approximately 70% confluency. Cells were then detached using 0.25% trypsin (MP Biomedical, Santa Ana, CA) and transferred to a 75-$cm^2$ flask with DMEM containing 5% FBS, 1x ITS, and 1x antibiotic only (1x AB; 100 U/ml penicillin and 100 µg/ml streptomycin). Upon confluency, cells were trypsinized and split into three 75-$cm^2$ flasks. Confluent third passage cells were then trypsinized and transferred to DMEM containing 20% FBS, 1x ITS, 1x AB/AM, and 10% dimethyl sulfoxide (DMSO; Thermo Fisher). Cells were cryopreserved in liquid nitrogen for future experiments.

### 3.3.4 Treatment of Dermal Fibroblasts with LPS and Heat-Killed *E. coli*

Two fibroblast challenges were performed within the study. First, we challenged all 60 cultures with LPS to determine the initial ranking. Second, to compare the side-by-side response of the animals that were selected for the intra-mammary *E. coli* treatment, we challenged fibroblasts from the 6 high-ranked and 6 low-ranked animals (see below) with LPS and heat-killed *E. coli*. In both challenges, cells were revived from cryopreservation into pre-warmed DMEM containing 5% FBS, 1x ITS, and 1x AB/AM, and cultured to confluency. Fourth passage cells were detached with trypsin and seeded in 6-well plates at a concentration of $7.5 \times 10^4$ cells/ml (challenge 1) or $1.25 \times 10^5$ cells/ml (challenge 2) in a total volume of 2.0 ml. After a 24-hour incubation, media was removed and replaced with fresh media only (negative control), or with media containing either 100 ng/ml of ultra-pure LPS isolated from *E. coli* O111:B4 (Millipore Sigma, St. Louis, MO) or heat-killed *E. coli* strain P4 at an MOI of 10.

To rank the whole cohort of 60 animals, cells were incubated for 24-hours in LPS and negative control media. To compare the 6 highs and 6 lows, cells were incubated for 0, 2, 8, and 24-hours with LPS and for 24-hours with heat-killed *E. coli.*

Media was collected from 0 hour cultures as the negative control. After 24-hours, challenge media was removed from all cells and stored at -20°C until further analysis. Cell lysate was also obtained by applying 800 µl of lysis buffer (PureLink™ RNA Mini Kit, Thermo Fisher) directly to the wells and stored at -80ºC until RNA extraction.

### 3.3.5 Quantification of IL-8 and IL-6 Protein in Challenge Media

Production of IL-8 in conditioned media was measured using a commercially available sandwich ELISA per manufacturer's instructions with slight modifications (Mabtech, Cincinnati, OH). Capture antibody was diluted to a concentration of 1 µg/ml in 0.05 *M* bi-carbonate buffer. Biotinylated detection antibody was diluted to 0.025 µg/ml in PBS-0.05% Tween-20. Streptavidin-horseradish peroxidase (Strep-HRP; Millipore Sigma) was diluted to a concentration of 0.07 µg/ml in PBS-0.05% Tween-20. Recombinant bovine IL-8 (Thermo Fisher) was used as the assay standard with a detection limit of 78.125 pg/ml.

Production of IL-6 was similarly measured by sandwich ELISA per manufacturer's instructions (R&D Systems, Minneapolis, MN). Recombinant bovine IL-6 (R&D Systems) was used as the assay standard with a detection limit of 156.25 pg/ml. For both assays, plate washing was performed 3 times with PBS-0.05% Tween-20 between each step. After the final plate wash, development of IL-8 and IL-6 assays was done by adding 3,3',5,5'-tetramethylbenzidine substrate (TMB; Thermo Scientific) and the reaction was stopped with 1 *M* $H_2SO_4$ after 3-5 minutes. Absorbance was measured at 450 nm with a plate reader (Synergy-HT, Bio-Tek, Winooski, VT) to quantify protein levels using a 4-parameter analysis with optical density corrected against blank wells.

### 3.3.6 Fibroblast Gene Expression

Extraction of RNA from cell lysate was done using the PureLink™ RNA Mini Kit (Thermo Fisher), which included a 15-minute DNase treatment that was performed using the PureLink™ DNase Set (Thermo Fisher). Concentration of RNA was determined using a Qubit™ 2.0 Fluorometer (Life Technologies, Carlsbad, CA) and first-strand cDNA synthesis was done using the Improm-II™ Reverse Transcriptase Kit (Promega, Madison, WI) on 400 – 600 ng of RNA. Gene expression analysis of select immune response genes was measured by real-time quantitative PCR (RT-qPCR) on a CFX96 Touch™ Real-Time PCR Detection System (BioRad, Hercules, CA) using Thermo Scientific™ Maxima™ SYBR™ Green/Fluorescein qPCR Master Mix (Thermo Fisher). Cycling conditions were as follows: 95°C for 2 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min and 72 °C for 1 min after which a melt curve was inserted. All oligonucleotide primer sequences are listed in Table 3.1. Unless already validated and published, primers were designed using the Primer 3 program on NCBI. Primers were designed to amplify a 100-300 base pair region without any known SNPs and if possible, to span an exon-intron junction and include a GC clamp. Primer sequences were then analyzed by the NCBI nucleotide BLAST program to ensure only the target *Bos taurus* gene was detected. Melt curve analysis of all primer sets was analyzed and only one peak could be detected, indicating a single amplicon without primer-dimer contamination. No amplification was detected in negative (no cDNA controls).

### 3.3.7 Preparation of *E. coli* for Intra-mammary Infection

An aliquot of cryopreserved *E. coli* strain P4, originally isolated from an acute case of clinical mastitis (Bramley, 1976) and classified as O32:H37, ECOR phylogenetic

group A and multilocus sequence type ST10 (Blum et al., 2012) was initially grown overnight on trypticase soy agar containing 5% sheep's blood (SBA; Northeast Laboratory Services, Waterville, ME) at 37ºC to isolate a single colony. One colony was then inoculated into 3 mL of trypticase soy broth (TSB; Thermo Fisher) and incubated at 37ºC with shaking for 6 hours. One mL of the 6-hour culture was then inoculated into 99 mL of TSB and incubated for 16 hours at 37ºC with shaking. The stock culture was kept at 4ºC until concentration of bacteria was determined by serial dilution and plating. Once determined, stock culture was diluted to 40 cfu/ml in sterile, endotoxin free, isotonic saline (Phoenix, St. Louis, MO) in preparation for intra-mammary challenge.

### 3.3.8 Experimental Mastitis Challenge

Twelve animals, determined to be either high (n=6) or low (n=6) responders based on their fibroblast phenotype, were chosen for an experimental mastitis challenge with *E. coli*. Fibroblast ranking was based primarily on baseline expression of TLR4 followed by cytokine response to LPS. Using this ranking strategy, 4 high responders (HR) and 4 low responders (LR) were within the top and bottom 20% of TLR4 expression, respectively. The 2 HR and 2 LR that were not within the 20% cutoff were chosen based on their ranking within the top or bottom 20% of IL-8 or IL-6 response and on their mastitis free status and availability from the collaborating farm.

All 12 animals were transported to the UVM Miller Research Farm and allowed to acclimate for 7 days before the infusion. Ten of the animals were pregnant and the 2 non-pregnant animals were maintained in diestrus using controlled internal drug release inserts containing progesterone (Eazi-Breed™ CIDR®, Zoetis US, Parsippany, NJ). Cows were milked twice daily at 12-hour intervals using individual milking units.

Quarter milk samples were taken prior to the challenge to ensure quarters were free of mastitis-causing pathogens and composite SCC was below 200,000 cells/ml. Following morning milking on the day of the challenge, teat ends were disinfected with 70% ethanol and 5 mL of the bacterial solution was infused into the right hind quarter using a teat cannula. The infusion solution was plated in triplicate and plate counting was done to determine the actual infusion dose, which was 37 cfu/ml and 185 cfu total. Following the intra-mammary challenge, rectal temperature was monitored for 96 hours. Blood samples were taken by venipuncture of the tail vein at 0, 12, 24 and 36 hours post-infection for serum cytokine analysis and at 7 and 14 days post infection to measure antibody response. Blood samples were collected in vacutainer tubes containing sodium-heparin (BD, Franklin Lakes, NJ) and transported to the lab on ice. Blood was centrifuged at 1,000 x *g* for 15 minutes at 4ºC. Plasma layer was removed and stored at -20ºC for downstream experiments.

Aseptic milk samples were taken at 12-hour intervals for 5 days, at 24-hour intervals for an additional 5 days and at 48-hour intervals for the remainder of the study. Bacterial clearance was measured from infected quarters by plating 10 µl of undiluted milk or milk diluted in sterile PBS onto SBA, incubating overnight at 37ºC, and counting colonies. Infected quarter SCC was measured using a portable cells counter (DCC, DeLaval, Tumba, Sweden). Infected quarter milk was collected using a separate quarter milker at each milk sampling time-point to measure infected quarter production.

### 3.3.9 Whey Preparation

Whole milk samples were centrifuged at 16,000 x *g* at 4°C for 30 minutes. Top cream layer was removed and skim milk samples were centrifuged again at 16,000 x *g* at

4°C for 30 minutes. The whey supernatant was removed and stored at -20°C for downstream analysis.

### 3.3.10 Measurement of Proteins in Whey

Concentrations of IL-8 and IL-6 in whey were measured as previously described. Whey was diluted 1:5 in PBS-0.05% Tween-20 for IL-8 assays and 1:10 in PBS-0.05% Tween-20 for IL-6 assays.

Bovine serum albumin (BSA) concentrations were measured per manufacturer's instructions using a commercially available ELISA kit (Bethyl Laboratories, Montgomery, TX) with slight modifications. Briefly, capture antibody was diluted 1:100 in 0.05 *M* bi-carbonate buffer to a concentration of 10 µg/ml and incubated at room temperature for 1 hour. Plates were blocked overnight at 4ºC with PBS-0.05% Tween-20. Whey samples were diluted 1:40,000 in PBS-0.05% Tween-20. Samples and standards were incubated at room temperature for 1 hour. Horseradish peroxidase-conjugated secondary antibody was diluted 1:50,000 in PBS-0.05% Tween-20 to a concentration of 20 ng/ml and incubated at room temperature for 1 hour. Plates were washed between each step 3 times with PBS-0.05% Tween-20. After the last wash step, plates were developed by adding TMB and allowed to develop for 5-7 minutes. The reaction was stopped by adding 1 *M* $H_2SO_4$ and absorbance was read at 450 nm with a plate reader as previously described. Detection limit of the BSA assay was 6.25 ng/ml.

### 3.3.11 Circulating Cytokine Response

Serum TNF-α levels were measured by radioimmunoassay (RIA) as previously described (Elsasser et al., 2005) and serum IL-6 was measured by ELISA as previously described.

### 3.3.12 Heat Killing Bacteria

A cryopreserved stock of the same *E. coli* P4 challenge strain was grown as described earlier for the intra-mammary challenge preparation. A plate dilution was set up to determine concentration of stock bacteria. Stock bacteria was then subjected to heat killing by incubating in a water bath at 70ºC for 45 minutes with shaking every 10 minutes. Heat killed bacteria was then stored at 4ºC overnight while 100 µl of heat killed bacteria was plated onto SBA and incubated overnight to ensure bacteria were killed. The following day, heat killed bacteria was spun at 3600 x *g* for 15 minutes at 4ºC and re-suspended in 50 mL of double distilled water. Heat killed bacteria was then aliquoted and stored at -20ºC for downstream applications.

### 3.3.13 Antibody Response to challenge strain

Total anti-*E. coli* IgG levels were measured in plasma at 0, 7 and 14 days post infection using a custom indirect ELISA. Heat killed *E. coli* was diluted 1:100 in 0.05 *M* bi-carbonate buffer to a concentration of 1 x $10^7$ heat-killed bacteria. Diluted *E. coli* and bi-carbonate buffer only (blank wells) were incubated overnight at 4ºC. Blocking was then performed for 1 hour at room temperature with PBS-2% non-fat milk (Lab Scientific, Highlands, NJ). Plasma was plated at a dilution of 1:1000 in PBS-0.05% Tween-20 and incubated at room temperature for 1 hour. Horseradish peroxidase-conjugated goat anti-bovine IgG was diluted 1:20,000 in PBS-0.05% Tween-20 to a concentration of 0.025 µg/ml and incubated at room temperature for 1 hour. Plates were washed three times with PBS-0.05% Tween-20 between each step. After the final plate wash, TMB was added and the reaction was allowed to develop for approximately 5

minutes. The reaction was stopped with 1 *M* $H_2SO_4$. Optical density at 450 nm was measured as previously described.

### 3.3.14 Statistical Analysis

An unpaired Mann-Whitney U test in GraphPad Prism 7.0 (GraphPad Software Inc., La Jolla, CA) was performed to compare concentrations of IL-8 and IL-6 in dermal fibroblast challenge media and to compare 12-hr. SCC response. Spearman rank correlation and linear regression analysis of IL-8 and IL-6 production in challenge media from LPS vs. heat-killed *E. coli* cultures was also performed in GraphPad. Analysis of dermal fibroblast gene expression, serum cytokine response and humoral response was done using a 2-way repeated measures ANOVA in GraphPad with time as the repeated measure to determine time x fibroblast response significance. A post-hoc Bonferroni's multiple comparisons test was then done to determine significance at each time point. Finally, comparison of phenotype (LR vs HR) on concentrations of whey IL-6, IL-8 and BSA, infected quarter milk production, rectal temperature and infected quarter SCC and bacteria counts, both transformed for normal distribution, were analyzed using a linear regression model assuming a first order auto-regressive covariance structure with time post-infection as the repeated measure. A least square means test was performed to determine effect of time, time x fibroblast response and to compare fibroblast response at each time point. This analysis was performed using the MIXED procedure in SAS version 9.4 (SAS Institute Inc., Cary, NC). All values are presented as the mean of each group ± SEM. Statistical significance is defined as $P<0.05$ and a trend as $P<0.10$.

## 3.4 Results

### 3.4.1 Dermal Fibroblast Response to LPS

The sixty fibroblast cultures were revived from cryopreservation in five separate batches of twelve cultures. The cells were mixed by sampling date, such that each challenge batch contained fibroblasts that were isolated on all four sampling dates. Unfortunately, 2 of the 60 cultures did not survive cryopreservation, leaving 58 LPS-challenged cultures.

Fibroblasts were treated with either media alone or media supplemented with 100 ng/ml of LPS for 24 hours. Fibroblast production of IL-8 and IL-6 were measured in all media samples following the challenge and were adjusted for final cell number. Levels of IL-8 and IL-6 in negative controls were minimal but were subtracted from the challenge values. In addition, to normalize for batch-to-batch variation between challenges, protein amounts were adjusted for batch mean and standard deviation. Adjusted rank, ranging from -1.50 to 2.33 for IL-8 and from -1.65 to 2.54 for IL-6, were used as response variables. As with previous experiments (Kandasamy et al., 2011, Benjamin et al., 2015b), a large range in response to LPS was measured in conditioned media, with a significant correlation between LPS-induced IL-6 and IL-8 production (data not shown) ($R^2 = 0.28$, $P<0.0001$).

### 3.4.2 Dermal Fibroblast Expression of *TLR4* and Ranking

Expression of *TLR4* was measured in cultures from negative, media-only control wells. Of the 58 challenged cultures, three cultures had very low RNA yield and were excluded from further ranking. Expression of *TLR4* was measured in the remaining 55 cultures using two primer sets that showed a 40- and 70-fold difference in expression

between the lowest and highest cultures. The two primer sets also exhibited a significant correlation in ranking of *TLR4* expression (data not shown) ($R^2 = 0.77$, $P<0.0001$). As such, measurement of *TLR4* will be presented using one of the two primer sets for the remainder of experiments.

Fibroblast phenotype was determined by sorting and ranking on all three variables as shown in figure 3.1. Selection was then based on identifying the tail ends of *TLR4* expression and whenever possible, choosing animals with a positive or negative rank for both IL-8 and IL-6 as our high and low responders, respectively. Using this strategy, 6 selected high responders (figure 3.1, red dots) and 6 selected low responders (figure 3.1, blue dots) were selected for subsequent in vitro and in vivo experiments.

### 3.4.3 Phenotype Confirmation of 12 Selected Animals

To confirm that our ranking strategy accurately predicts fibroblast response to LPS and to compare whether LPS and heat-killed *E. coli* would elicit a similar response, fibroblasts from the 12 selected highs and lows were revived a second time from cryopreservation and treated for 24 hours with media alone, media supplemented with 100 ng/ml of LPS, and media supplemented with heat-killed *E. coli* at an MOI of 10.

As predicted, LPS-induced production of IL-6 protein was higher in animals ranked as high responders versus those ranked as low responders (figure 3.2, panel A). High responding fibroblasts produced 32.5 ± 3.3 fg/cell of IL-6 as compared to low responders that produced 20.2 ± 3.9 fg/cell. Interestingly, this difference did not extend to fibroblast response to heat-killed *E. coli*, even though LPS and heat-killed *E. coli* production of IL-6 was significantly correlated ($R^2=0.76$, $P<0.01$) within animals (figure 3.2, panel C). No detectable IL-6 was measured in fibroblasts cultured with media alone.

Similar results were measured in LPS-induced production of IL-8. High responding cultures produced significantly more IL-8 in response to LPS in comparison to low responding cultures (2.51 ± 0.2 vs 1.4 ± 0.1 fg/cell, $P<0.001$, figure 3.2, panel B). However, IL-8 production was also significantly elevated in high responding cultures in response to heat-killed *E. coli* (1.8 ± 0.1 vs 1.0 ± 0.1 fg/cell, $P<0.05$, figure 3.2, panel B). Fibroblast cultures that were treated with media alone did have low background levels of IL-8 and notably, these levels were significantly elevated (0.25 ± 0.1 vs. 0.1 ± 0.04 fg/cell, $P<0.001$) in high responding cultures but did not affect differences in response to LPS or heat-killed *E. coli* (figure 3.2, panel B). As with IL-6 production, fibroblast production of IL-8 is significantly correlated ($R^2=0.61$, $P<0.01$) within-animals when comparing response to LPS and to heat-killed *E. coli* (figure 3.2, panel D). Fibroblasts also produce more IL-8 in response to LPS than to heat-killed *E. coli*, the opposite of which is true for fibroblast production of IL-6.

### 3.4.4 Fibroblast Gene Expression in Response to LPS

To determine whether protein differences would also be reflected at the mRNA level and to measure the expression of other innate response genes, gene expression analysis was performed on high and low responding fibroblasts at 0 (control), 2, and 8 hours post LPS treatment. Expression of both *IL-8* and *IL-6* were up-regulated by 2 hours post LPS treatment and were significantly higher ($P<0.05$, figure 4.3, panels A & B) in high responding cultures, confirming the protein response difference. The largest difference in *IL-8* and *IL-6* expression between the two sets of cultures was measured at 2-hrs post LPS at which time high responding cultures exhibited a 4.6- and 4.8-fold higher level of expression, respectively. Interestingly, difference in *TLR4* expression was

not as high as expected based on our initial ranking, perhaps due to technical variation of the RT-qPCR assay. However, expression of *TLR4* was higher in high responding cultures with a time x response difference that trended towards significance ($P<0.10$, figure 3.3 panel C). Again, the greatest difference between the two sets of cultures was measured at 2-hrs. post LPS treatment, at which time a significant ($P<0.05$) 2-fold higher level of expression was seen in high responding cultures. There was also a small (3.5-fold), but significant increase in *TLR4* expression by 8-hrs post-LPS treatment.

The expression of three other innate immune response genes were also measured to determine whether the high and low phenotype was unique to our ranking variables. The expression of *TNF-α* was measured as this is a crucial early-response cytokine with a similar, but not identical role in the innate response as *IL-6*. As expected, a large increase in *TNF-α* expression was measured by 2-hrs. post LPS treatment that returned close to baseline by 8 hrs (figure 3.3, panel D). Overall, there was a time x response trend towards significance ($P<0.10$) for higher expression in high responding cultures. As with the previously measured genes, the greatest difference was measured at 2-hrs. post LPS at which time high responding cultures exhibited a 2-fold higher level of *TNF-α* expression. Expression of serum amyloid A-3, a member of the SAA family of acute phase proteins, was also measured in response to LPS treatment. Although expression was higher in high responding cultures at all time points, no significant differences were measured between the two groups of animals (figure 3.3, panel E). Finally, a large increase in expression of *CCL20*, a CC-family chemokine, was measured in response to LPS treatment, with no differences observed between high and low responding cultures (figure 3.3, panel F).

### 3.4.5 Intra-mammary Infection with *E. coli*

To determine how dermal fibroblast phenotype relates to *in vivo* response, the 6 high responders and 6 low responders were given an infusion of 185 cfu of *E. coli* in their right-hind quarter following morning milking. Clinical signs of coliform mastitis, such as lethargy, fever and anorexia, were evident by 12-hrs. post infection. One high responding animal developed acute toxic mastitis and was humanely euthanized at 48 hrs. post infection. Her data was utilized for milk samples up to 12-hrs. post infection and rectal temperatures and blood samples up to 36-hrs. post infection. Rectal temperatures were monitored for 96-hrs. post-infection and peaked at 15-hrs. in all animals (figure 3.4), indicative of an acute case of mastitis, and returned to baseline by 21-hrs. post-infection. Overall, there was no difference in febrile response between high and low responders, however, high responders had an early and significant increase ($39.4 \pm 0.2$ vs $38.7 \pm 0.2$ºC, $P<0.05$) in temperature by 12-hrs. post-infection in comparison to low responding animals, suggesting that high responders may react more quickly to *E. coli* infection. In contrast, low responders had a significantly elevated temperature at 18-hrs. post-infection ($39.4 \pm 0.4$ vs $38.8 \pm 0.3$ºC, $P<0.05$). This result should be taken with caution, however, as a non-steroidal anti-inflammatory (Banamine®, flunixin meglumine) was administered to half of the animals (4 HR and 2 LR) at 15-hrs. post-infection per IACUC requirements. All temperatures returned to baseline by 21-hrs. post-infection.

A notable drop in infected (figure 3.5) and uninfected (data not shown) quarter milk production was measured by 24-hrs. post infection. No difference in post-infection milk production was measured in the high versus low responding animals. All animals recovered to pre-infection milk production levels by the end of the study, with the

exception of one low responder that stayed close to dry-off from 144-hrs post-infection until the end of the study and one high responder that had very low production on the last day of sampling due to re-infection in her right hind quarter.

Leukocyte influx into the mammary gland was assessed by measuring increase in infected quarter somatic cell count (SCC) for 14 days following *E. coli* infection (figure 3.6, panel A). Using a linear regression model, there was no difference in SCC between high and low responders, with the exception of a trend towards elevated SCC in high responders at 60 and 108-hrs. post infection. Interestingly, it was noted that at 12-hrs. post infection high responders had already increased their SCC above baseline, whereas low responders remained near pre-infection levels. In light of this observation, we performed an assessment of just the increase in SCC at 12-hrs. post infection (figure 3.6, panel B) that showed that high responders had an up-regulated SCC that was significantly (183,000 ± 98,000 vs. 26,000 ± 11,000 SCC/ml, $P<0.05$) higher than low responders at this early time point post-infection. The difference in SCC was gone by 24-hrs. post infection, but in conjunction with their elevated febrile response, may indicate that high responding animals respond more quickly to infection. Somatic cell count remained elevated in all animals through the end of the study.

Recovery of *E. coli* from the infected gland was similarly measured for 14 days post-infection to determine how phenotype impacts bacterial clearance. As shown in figure 3.6 panel C, bacteria quickly multiply post-infection, reaching maximal levels at 12-24-hrs. post-infection. Interestingly, while high responding animals peak at 12-hrs post-infection, levels of bacteria keep increasing in low responding animals until 24-hrs. post infection. Over the entire course of infection, high responding animals trended

(P=0.10) towards lower bacterial levels and enhanced bacterial clearance which reached significance (P<0.05) at 24, 72, and 84 hours post infection. All animals were clear of infection by 12 days post infection, however, one high responding animal became re-infected with *E. coli* at 14 days post infection.

### 3.4.6 Infected Quarter Cytokine Response and Tissue Damage

Infected quarter whey concentrations of IL-8, IL-6 and bovine serum albumin (BSA) were measured to determine how phenotype affects local inflammatory response and tissue damage. A dramatic increase in IL-8 was measured by 24-hrs. post infection (figure 3.7, panel A) that returned to baseline by 96-hrs. post infection. Overall, there was no difference between high and low responders in infected quarter levels of IL-8, however, high responding animals had significantly (27.8 ± 5.9 vs. 18.5 ± 3.5 ng/ml, P<0.05) higher concentrations of IL-8 at 24-hrs. post-infection. This difference at 24-hrs. may partially contribute to the higher SCC levels measured at 12-hrs. post-infection in high responding animals as IL-8 is the primary chemokine resulting in neutrophil chemotaxis.

Whey concentrations of IL-6 also peaked at 24-hrs. post-infection, however no differences were measured between high and low responders at any time point post-infection (figure 3.7, panel B). Levels of IL-6 returned to baseline by 144-hrs. post-infection, indicating IL-6 has a prolonged role in response to *E. coli* infection in comparison to IL-8. Whey concentrations of BSA, an indicator of blood-milk barrier damage, were again significantly elevated in all animals by 24-hrs. post-infection and, with the exception of one animal, returned to baseline by 144-hrs. post-infection (figure 3.7, panel C). One low responder had elevated levels of BSA and very low milk yield

until 216-hrs. post-infection due to persistence of the *E. coli* infection. No significant differences in BSA levels were measured between high and low responders at any time point post-infection.

### 3.4.7 Systemic Response to *E. coli* Infection

Concentrations of plasma TNF-α and IL-6 were measured every 12 hrs. for 36 hrs. following the infection as markers of systemic response to *E. coli*. Peak TNF-α levels were detected at 24-hrs. post-infection, with no significant difference between low and high responders, overall. A trend towards higher levels of TNF-α in low responders was detected at 24-hrs. post-infection ($0.13 \pm 0.03$ vs $0.09 \pm 0.02$ ng/ml, $P<0.10$) (figure 3.8, panel A). Concentrations of plasma IL-6 was also highest at 24-hrs, with no significant differences measured between high and low responding animals (figure 3.8, panel B).

### 3.4.8 Humoral Response to *E. coli* Infection

Finally, to determine if levels of total anti-*E. coli* serum IgG would differ by fibroblast phenotype following infection, blood samples were taken to measure antibody response at 0, 7 and 14 days post-infection. A significant increase in *E. coli*-specific IgG was measured by day 7 that remained elevated through day 14, however, no difference was measured between the high and low responding animals (figure 3.9).

## 3.5 Discussion

In the current analysis, we aimed to refine the high and low fibroblast phenotype based on earlier, promising results that animals whose fibroblasts produce more IL-8 in response to synthetic TLR agonists, LPS and PAM2CSK4, have a more severe response to *E. coli* and *S. aureus* mastitis, respectively. If able to accurately predict cow response

to infection, the dermal fibroblast could be used as a valuable tool to discover genetic or epigenetic markers that cause resistance or susceptibility to severe mastitis.

In addition to increased profitability for the dairy producer, selection of replacement heifers based on genetic resistance to mastitis has shown increased interest due to an effort to reduce antibiotic usage in food-producing animals and to improve animal welfare. Genetic variability that results in increased susceptibility or severity of infection has already been observed in various species in response to a wide range of pathogens. In one report, a polymorphism in the *IL17A* gene was shown to lead to increased susceptibility to Gram-positive infection and sepsis mortality (Nakada et al., 2011). Copy number variants are another, less common type of gene variant that have been shown to contribute to differences in susceptibility to malaria and HIV infection in humans (Hollox and Hoh, 2014) and are also gaining attention in agriculture species as another potential source of variation in mastitis susceptibility (Duran Aguilar et al., 2017). Genetic differences in *CXCR1*, the main receptor for IL-8 signaling, was also recently shown to impact severity of experimental intra-mammary infection with *Streptococcus uberis*, whereby a haplotype was discovered to be protective against both inflammation and bacterial growth (Siebert et al., 2017).

Using a slightly different approach, our hypothesis was that mastitis severity would be reflected in the fibroblast innate response phenotype, such that fibroblasts could be utilized in the future to identify the genetic or epigenetic basis of severe mastitis resistance and/or susceptibility using genome-wide sequencing techniques. The decision to use dermal fibroblasts over other cell culture models was based on their ease of isolation, ability to cryopreserve, responsiveness to TLR agonists, low baseline

activation, and on previous research suggesting that the primary fibroblast accurately reflects progression of natural infection. Primary dermal fibroblasts have been used as a model cell to identify strain-specific differences in the virulence of Herpesvirus sub-species in infections of non-natural hosts (Rogers et al., 2007) and in cattle to mimic pathogen-specific differences in mammary gland response to mastitis (Gunther et al., 2016b). Furthermore, mammary-derived stromal fibroblasts (BMFs) isolated from *E. coli*-infected versus healthy cows exhibit a pro-inflammatory phenotype at isolation and are able to induce epithelial cell expression of *TNF-α* and *IL-8* in a co-culture system (Chen et al., 2016). Conversely, epithelial cells pre-treated with LPS or lipoteichoic acid (LTA) are able to induce pro-inflammatory gene expression in BMFs isolated from healthy cattle (Zhang et al., 2016). Both studies are evidence of the cross-talk that occurs between the epithelial cell and fibroblast within the mammary gland and the importance of the fibroblast in initial response to infection.

In the current study, primary bovine dermal fibroblasts were isolated from ear notch biopsies and used to study the cow-to-cow variation in mastitis severity that exists independent of pathogen type and host physiological state. Fibroblasts were initially isolated from a cohort of 60 multiparous, adult Holsteins at the same stage in lactation, and their fibroblast phenotype was ranked from high to low innate response based on LPS-induced IL-8 and IL-6 protein production and *TLR4* gene expression. Six high responders and six low responders were then chosen, primarily on differences in *TLR4* gene expression and subsequently on their high or low production of both IL-8 and IL-6, for additional experiments. Since fibroblast response was measured in different batches, high and low cultures were re-challenged side-by-side and their difference in LPS-

induced production of IL-8 and IL-6 was confirmed. Bacterial endotoxin, or LPS, has been used in countless studies to mimic the response to *E. coli* and other Gram-negative bacteria, however, *E. coli* can also activate the innate response through receptors other than TLR4, such as TLR5 that is activated by flagellin (Andersen-Nissen et al., 2007), TLR9 that is activated by bacterial DNA (Magnusson et al., 2007), and DAP (D-glutamyl-meso-diaminopimelic acid)-dependent NOD1 activation (Kumar et al., 2011). As such, in the second challenge, we stimulated fibroblasts isolated from high and low responders with heat-killed *E. coli* along with LPS and showed that LPS-induced IL-8 production, and to a lesser extent IL-6 production, corresponds to response to *E. coli*. Fibroblast IL-8 production is also higher in response to LPS than to *E. coli*, the opposite of which is true for IL-6 that is produced in higher quantities in response to *E. coli*. The IL-8/IL-6 discrepancy could be due to increased signaling pathways that induce IL-6 production following *E. coli* stimulation or in differential post-transcriptional regulatory mechanisms such as mRNA stabilization that is shown to occur through interaction with Arid5a for *IL-6* transcripts (Nyati et al., 2017) and through p38 MAPK kinase and MK2 for *IL-8* transcripts (Bhattacharyya et al., 2011, Moretto et al., 2012).

LPS-induced gene expression was then measured in high and low responding fibroblasts to confirm that mRNA also reflects protein level differences and to measure the expression of other important innate response genes. As expected, mRNA levels of *IL-8, IL-6,* and *TLR4* were significantly higher in high responding cultures at 2-hrs. post LPS stimulation. We were, however, surprised that baseline (0-hr.) *TLR4* expression only trended towards significance with a 1.9-fold higher level of expression in high responders, when in our initial ranking high responders exhibited a significant 6.9-fold

higher level of expression using identical primer sets. In addition to inherent between-assay variation, this discrepancy could be explained by length of cryopreservation and by differences in initial cell density between the two sets of experiments. Expression of *TNF-α* also trended towards higher levels in high responding cultures. This difference is somewhat expected based on the role of TNF-α as a canonical pro-inflammatory and pleiotropic cytokine with similar regulatory mechanisms to IL-6. Serum amyloid a3 (*SAA3*), an acute-phase protein that is induced by pro-inflammatory cytokines TNF-α, IL-1ß and IL-6 following infection (Ye and Sun, 2015), was also measured post-LPS treatment to determine if differences in fibroblast IL-6 production would be reflected in *SAA3* expression. Although expression of *SAA3* was higher in high responders at all three time points, no significant differences were measured, and the weak induction of *SAA3* post-LPS stimulation suggests that more time may be required following LPS treatment for IL-6 to elicit its effect on *SAA3* expression. Finally, expression of *CCL20*, a CC-motif chemokine that is induced in response to *E. coli* mastitis (Petzl et al., 2012) and has also been shown to differ in expression in two other groups of high and low responding fibroblasts (Green et al., 2015, Benjamin et al., 2016a), was measured. The chemokine was highly up-regulated by 2-hrs. post LPS-treatment and overall, expression was higher in high responding cells, but again the difference did not reach significance.

With the high and low fibroblast phenotype established, all 12 animals were infected with the P4 strain of *E. coli* in their right-hind quarter to test their immune response to infection. Overall, the response to intra-mammary challenge with *E. coli* followed a similar magnitude and kinetics as has been noted in previous challenges with *E. coli* (Bannerman et al., 2008, Kandasamy et al., 2011) and were reflective of acute,

clinical mastitis. Unfortunately, very few differences were measured between the high and low responding animals and in general, the responses were extremely variable between all 12 animals. At the most severe, one high responder developed acute toxic mastitis first evidenced by dysstasia at 24-hrs post-infection, which resulted in our decision to humanely euthanize the animal at 48-hrs post-infection, as she did not respond to treatment with Banamine® and systemic antibiotic therapy. Another low responding animal completely dried off in her infected quarter at 192-hrs. post-infection and remained very low in infected quarter milk production until the end of the study. In general, milk production was drastically reduced by 24-hrs. post-infection in all animals with no differences measured between high and low responders. Again, there was significant animal-to-animal variation in their time to recovery to pre-infection milk production, ranging from 5 to >14 days post-infection. Increase in somatic cell count, mainly due to neutrophil recruitment into the mammary gland, tended to occur faster in high responding animals. High responders already had an elevated SCC at 12-hrs. post-infection, whereby it took low responders until 24-hrs. post infection to see a significant SCC increase. The role of the neutrophil as a beneficial first-line responder to clear bacteria versus that of a main source of inflammatory-related damage through the production of ROS, proteases and NET formation in the mammary gland has been the subject of debate (Aitken et al., 2011). Efficient leukocyte influx into the mammary gland is, however, thought to be beneficial for mastitis outcome (Burvenich et al., 2007) and in the current study we show that early up-regulation of SCC does occur with enhanced bacterial clearance. High responding animals that had an elevated SCC at 12-hrs. had lower overall bacterial counts throughout the study, and this difference reached

significance at several time points. Notably, while high responders peaked in their cfu/ml of bacteria by 12-hrs., concurrent with their elevated SCC, cfu/ml of *E. coli* continued to increase until 24-hrs. post infection in low responders. The difference in SCC was abolished by 24-hrs., remaining at similar levels between the two groups for the remainder of the study, and at 2-weeks post-infection SCC was still up-regulated in all animals in comparison to pre-infection levels. There is the potential that if SCC were measured for a longer period of time post-infection differences could be detected and perhaps longer sampling time should be considered for future experiments.

Interestingly, in addition to higher SCC at 12-hrs. post-infection, high responders had a significantly elevated febrile response at 12-hrs. in comparison to low responders, and both groups peaked at 15-hrs. post-infection before returning to baseline at 21-hrs. Although hyperpyrexia can be detrimental to the host, the febrile response is an ancient and conserved innate defense to infection and is likely another factor that limited bacterial growth in high responders (Cavaillon, 2012). Serum cytokine levels of TNF-α and IL-6, two of the three main pyrogenic cytokines, did not correlate with fever response, however, this may be due to the timing of serum collection and to the administration of Banamine® or flunixin meglumine, a non-steroidal anti-inflammatory. Flunixin inhibits the activity of cyclooxygenase enzymes that synthesize prostaglandin ($PGE_2$) from arachidonic acid, a process known to affect the innate response and systemic cytokine production (Kalinski, 2012). The NSAID was administered to 7 of the 12 animals (4 HR and 3 LR) between 15-18-hrs. post-infection, in accordance with animal welfare protocols, and represents one of the major but necessary limitations of the current study.

No differences were detected between high and low responders in the mammary gland proteins that were measured, IL-8, IL-6 and BSA. Nevertheless, a comparison of the area under the curve of milk cytokines versus milk BSA showed a significant correlation (Figure 3.10) in their production over the duration of the infection, such that animals with a prolonged cytokine response also exhibited more tissue damage. Concentration of IL-8 was also significantly higher in high responders at 24-hrs. post-infection and likely contributes to the early elevation of milk SCC in high responders at 12-hrs. Unfortunately, the lack of any real difference in milk IL-6 and IL-8 indicates that the fibroblast model may not accurately reflect the mammary gland environment, contradicting the results of our previous studies. The strength and duration of pro-inflammatory cytokine and chemokine production in the mammary gland is, however, directly related to tissue damage. Follow-up experiments should focus on the initial response to infection, likely mediated by resident epithelial and immune cells, as this response seems to be crucial for efficient leukocytosis and bacterial clearance. In addition, more emphasis should be placed on down-regulation of the inflammatory response as the majority of animals had a sufficient response to clear bacteria, but those that did not recover as well tended to have a sustained inflammatory response. Interleukin-10, an anti-inflammatory cytokine that exerts its negative effect on pro-inflammatory cytokine production through STAT3-dependent upregulation of SOCS3 (Mosser and Zhang, 2008), is also up-regulated in response to mastitis-related infections, however, the role of IL-10 and other anti-inflammatory proteins in between-animal variation in mastitis severity has not been established. Proteins, such as WISP-1 and IL-22 have been shown to mediate epithelial cell recovery and proliferation following

mechanical or infection related injury (Pociask et al., 2013, Quiros et al., 2017), a response that would be crucial to the recovery of milk-producing cells that become damaged during mastitis infection. The ability of the animal to clear apoptotic cells that accumulate following infection could also affect recovery efforts since apoptotic cells will become necrotic if not efficiently cleared, resulting in chronic autoimmune-related inflammation (Munoz et al., 2017).

Finally, no difference was measured between high and low responders in their *E. coli*-specific circulating IgG response and importantly, antibody response did not correlate with ability to clear infection. This result agrees with previous literature stating that antibody response is not a major contributor to *E. coli* or *S. aureus* clearance within the mammary gland (Benjamin et al., 2015b, Herry et al., 2017). The role of the adaptive response in determining between-cow differences in mastitis severity and duration should be the subject of future research, specifically the role of Th-17 cells, which have been shown to be the predominant T-cell responder in *E. coli* mastitis (Porcherie et al., 2016) and are associated with a primarily neutrophilic response. However, other immune cell types whose functions remain completely unknown in bovine mastitis, such as innate lymphoid cells that are present in high numbers in mucosal tissue and γδ T cells that are expressed at much higher levels in the bovine than other species, may also contribute to differences in mastitis severity.

The current study shows that while the primary dermal fibroblast derived from ear notch tissue is highly responsive to TLR agonists, the magnitude in innate responsiveness does not correlate with severity of *E. coli* mastitis. This result contradicts previous experiments in our lab where high responding animals, based on fibroblast IL-8

production, develop more severe mastitis when challenged with either *E. coli* or *S. aureus*. The main difference between the two studies was the location of fibroblast isolation. Previously, fibroblasts were isolated through a shoulder biopsy, whereas in the current experiment fibroblasts were isolated using an ear punch biopsy. It is possible that cellular phenotype in fibroblasts isolated from shoulder biopsies more accurately reflect mammary gland cellular response. Future experiments should consider reevaluating whether the fibroblast is a useful model for response to mastitis and if location of isolation affects cellular response. Cellular phenotype in the current study did reflect an ability to more effectively clear bacteria from the mammary gland, as evidenced by high responders having lower bacterial burden throughout the trial. It is tempting to speculate that this difference in bacterial clearance is due to an earlier increase in leukocyte influx into the mammary gland, but this result should be confirmed in follow-up studies. Finally, as has been observed in both natural and experimental mastitis, significant animal-to-animal variation was noted in the current experiment, whereby some animals recovered very quickly from a milder infection, whereas others experienced severe pathology and never recovered from infection. While it is known that in severe cases, SCC, cytokine levels and tissue damage are elevated, and that milk production is inhibited, the underlying factors that determine mastitis severity remain elusive and are reflective of our relative lack of understanding of the optimal immune response to mastitis. Future studies should aim to identify the precise cellular and molecular components that determine a rapid, efficient and effective response that clears infection while limiting collateral damage to mammary gland tissue.

## 3.6 References


Aitken, S. L., C. M. Corl, and L. M. Sordillo. 2011. Immunopathology of mastitis: insights into disease recognition and resolution. J Mammary Gland Biol Neoplasia 16(4):291-304.

Alain, K., N. A. Karrow, C. Thibault, J. St-Pierre, M. Lessard, and N. Bissonnette. 2009. Osteopontin: an early innate immune marker of Escherichia coli mastitis harbors genetic polymorphisms with possible links with resistance to mastitis. BMC Genomics 10:444.

Andersen-Nissen, E., T. R. Hawn, K. D. Smith, A. Nachman, A. E. Lampano, S. Uematsu, S. Akira, and A. Aderem. 2007. Cutting edge: Tlr5-/- mice are more susceptible to Escherichia coli urinary tract infection. J Immunol 178(8):4717-4720.

Bannerman, D. D., A. C. Kauf, M. J. Paape, H. R. Springer, and J. P. Goff. 2008. Comparison of Holstein and Jersey innate immune responses to Escherichia coli intramammary infection. Journal of dairy science 91(6):2225-2235.

Benjamin, A. L., B. B. Green, B. A. Crooker, S. D. McKay, and D. E. Kerr. 2016. Differential responsiveness of Holstein and Angus dermal fibroblasts to LPS challenge occurs without major differences in the methylome. BMC Genomics 17(1):258.

Benjamin, A. L., B. B. Green, L. R. Hayden, J. W. Barlow, and D. E. Kerr. 2015. Cow-to-cow variation in fibroblast response to a toll-like receptor 2/6 agonist and its relation to mastitis caused by intramammary challenge with Staphylococcus aureus. Journal of dairy science.

Bhattacharyya, S., U. Gutti, J. Mercado, C. Moore, H. B. Pollard, and R. Biswas. 2011. MAPK signaling pathways regulate IL-8 mRNA stability and IL-8 protein expression in cystic fibrosis lung epithelial cell lines. Am J Physiol Lung Cell Mol Physiol 300(1):L81-87.

Blum, S. E., E. D. Heller, S. Sela, D. Elad, N. Edery, and G. Leitner. 2015. Genomic and Phenomic Study of Mammary Pathogenic Escherichia coli. PLoS One 10(9):e0136387.

Bougarn, S., P. Cunha, F. B. Gilbert, A. Harmache, G. Foucras, and P. Rainard. 2011. Staphylococcal-associated molecular patterns enhance expression of immune defense genes induced by IL-17 in mammary epithelial cells. Cytokine 56(3):749-759.

Bramley, A. J. 1976. Variations in the susceptibility of lactating and non-lactating bovine udders to infection when infused with Escherichia coli. J Dairy Res 43(2):205-211.

Brand, B., A. Hartmann, D. Repsilber, B. Griesbeck-Zilch, O. Wellnitz, C. Kuhn, S. Ponsuksili, H. H. Meyer, and M. Schwerin. 2011. Comparative expression profiling of E. coli and S. aureus inoculated primary mammary gland cells sampled from cows with different genetic predispositions for somatic cell score. Genet Sel Evol 43:24.

Burvenich, C., D. D. Bannerman, J. D. Lippolis, L. Peelman, B. J. Nonnecke, M. E. Kehrli, Jr., and M. J. Paape. 2007. Cumulative physiological events influence the inflammatory response of the bovine udder to Escherichia coli infections during the transition period. Journal of dairy science 90 Suppl 1:E39-54.

Cavaillon, J. M. 2012. Good and bad fever. Crit Care 16(2):119.

Chen, Q., G. He, W. Zhang, T. Xu, H. Qi, J. Li, Y. Zhang, and M. Q. Gao. 2016. Stromal fibroblasts derived from mammary gland of bovine with mastitis display inflammation-specific changes. Sci Rep 6:27462.

Chow, J. C., D. W. Young, D. T. Golenbock, W. J. Christ, and F. Gusovsky. 1999. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. J Biol Chem 274(16):10689-10692.

Duran Aguilar, M., S. I. Roman Ponce, F. J. Ruiz Lopez, E. Gonzalez Padilla, C. G. Vasquez Pelaez, A. Bagnato, and M. G. Strillacci. 2017. Genome-wide association study for milk somatic cell score in holstein cattle using copy number variation as markers. J Anim Breed Genet 134(1):49-59.

Elsasser, T. H., J. W. Blum, and S. Kahl. 2005. Characterization of calves exhibiting a novel inheritable TNF-alpha hyperresponsiveness to endotoxin: associations with increased pathophysiological complications. J Appl Physiol (1985) 98(6):2045-2055.

Green, B. B., S. D. McKay, and D. E. Kerr. 2015. Age dependent changes in the LPS induced transcriptome of bovine dermal fibroblasts occurs without major changes in the methylome. BMC Genomics 16:30.

Gunther, J., M. Koy, A. Berthold, H. J. Schuberth, and H. M. Seyfert. 2016. Comparison of the pathogen species-specific immune response in udder derived cell types and their models. Vet Res 47:22.

Herry, V., C. Gitton, G. Tabouret, M. Reperant, L. Forge, C. Tasca, F. B. Gilbert, E. Guitton, C. Barc, C. Staub, D. G. E. Smith, P. Germon, G. Foucras, and P. Rainard. 2017. Local immunization impacts the response of dairy cows to Escherichia coli mastitis. Sci Rep 7(1):3441.

Hollox, E. J. and B. P. Hoh. 2014. Human gene copy number variation and infectious disease. Hum Genet 133(10):1217-1233.

Kalinski, P. 2012. Regulation of immune responses by prostaglandin E2. J Immunol 188(1):21-28.

Kandasamy, S., B. B. Green, A. L. Benjamin, and D. E. Kerr. 2011. Between-cow variation in dermal fibroblast response to lipopolysaccharide reflected in resolution of inflammation during Escherichia coli mastitis. Journal of dairy science 94(12):5963-5975.

Kumar, H., T. Kawai, and S. Akira. 2011. Pathogen recognition by the innate immune system. Int Rev Immunol 30(1):16-34.
Lee, J. W., D. D. Bannerman, M. J. Paape, M. K. Huang, and X. Zhao. 2006. Characterization of cytokine expression in milk somatic cells during intramammary infections with Escherichia coli or Staphylococcus aureus by real-time PCR. Vet Res 37(2):219-229.

Leimbach, A., A. Poehlein, J. Vollmers, D. Gorlich, R. Daniel, and U. Dobrindt. 2017. No evidence for a bovine mastitis Escherichia coli pathotype. BMC Genomics 18(1):359.
Lu, Y. C., W. C. Yeh, and P. S. Ohashi. 2008. LPS/TLR4 signal transduction pathway. Cytokine 42(2):145-151.

Magnusson, M., R. Tobes, J. Sancho, and E. Pareja. 2007. Cutting edge: natural DNA repetitive extragenic sequences from gram-negative pathogens strongly stimulate TLR9. J Immunol 179(1):31-35.

Moretto, N., S. Bertolini, C. Iadicicco, G. Marchini, M. Kaur, G. Volpi, R. Patacchini, D. Singh, and F. Facchinetti. 2012. Cigarette smoke and its component acrolein augment IL-8/CXCL8 mRNA stability via p38 MAPK/MK2 signaling in human pulmonary cells. Am J Physiol Lung Cell Mol Physiol 303(10):L929-938.

Mosser, D. M. and X. Zhang. 2008. Interleukin-10: new perspectives on an old cytokine. Immunol Rev 226:205-218.

Munoz, L. E., M. Leppkes, T. A. Fuchs, M. Hoffmann, and M. Herrmann. 2017. Missing in action-The meaning of cell death in tissue damage and inflammation. Immunol Rev 280(1):26-40.

Nakada, T. A., J. A. Russell, J. H. Boyd, and K. R. Walley. 2011. IL17A genetic variation is associated with altered susceptibility to Gram-positive infection and mortality of severe sepsis. Crit Care 15(5):R254.

Nyati, K. K., K. Masuda, M. M. Zaman, P. K. Dubey, D. Millrine, J. P. Chalise, M. Higa, S. Li, D. M. Standley, K. Saito, H. Hanieh, and T. Kishimoto. 2017. TLR4-induced NF-kappaB and MAPK signaling regulate the IL-6 mRNA stabilizing protein Arid5a. Nucleic Acids Res 45(5):2687-2703.

Pareek, R., O. Wellnitz, R. Van Dorp, J. Burton, and D. Kerr. 2005. Immunorelevant gene expression in LPS-challenged bovine mammary epithelial cells. J Appl Genet 46(2):171-177.

Petzl, W., J. Gunther, T. Pfister, C. Sauter-Louis, L. Goetze, S. von Aulock, A. Hafner-Marx, H. J. Schuberth, H. M. Seyfert, and H. Zerbe. 2012. Lipopolysaccharide pretreatment of the udder protects against experimental Escherichia coli mastitis. Innate Immun 18(3):467-477.

Pociask, D. A., E. V. Scheller, S. Mandalapu, K. J. McHugh, R. I. Enelow, C. L. Fattman, J. K. Kolls, and J. F. Alcorn. 2013. IL-22 is essential for lung epithelial repair following influenza infection. Am J Pathol 182(4):1286-1296.
Porcherie, A., F. B. Gilbert, P. Germon, P. Cunha, A. Trotereau, C. Rossignol, N. Winter, P. Berthon, and P. Rainard. 2016. IL-17A Is an Important Effector of the Immune Response of the Mammary Gland to Escherichia coli Infection. J Immunol 196(2):803-812.

Quiros, M., H. Nishio, P. A. Neumann, D. Siuda, J. C. Brazil, V. Azcutia, R. Hilgarth, M. N.
O'Leary, V. Garcia-Hernandez, G. Leoni, M. Feng, G. Bernal, H. Williams, P. H. Dedhia, C. Gerner-Smidt, J. Spence, C. A. Parkos, T. L. Denning, and A. Nusrat. 2017. Macrophage-derived IL-10 mediates mucosal repair by epithelial WISP-1 signaling. J Clin Invest 127(9):3510-3520.

Rogers, K. M., D. H. Black, and R. Eberle. 2007. Primary mouse dermal fibroblast cell cultures as an in vitro model system for the differential pathogenicity of cross-species herpesvirus papio 2 infections. Arch Virol 152(3):543-552.

Rollin, E., K. C. Dhuyvetter, and M. W. Overton. 2015. The cost of clinical mastitis in the first 30 days of lactation: An economic modeling tool. Prev Vet Med 122(3):257-264.

Sheridan, M. P., G. Regev-Shoshani, J. Martins, S. Vimalanathan, and C. Miller. 2016. Nitric oxide modulates the immunological response of bovine PBMCs in an in vitro BRDc infection model. Res Vet Sci 109:21-28.

Siebert, L., S. Headrick, M. Lewis, B. Gillespie, C. Young, L. Wojakiewicz, O. Kerro-Dego, M. E. Prado, R. Almeida, S. P. Oliver, and G. M. Pighetti. 2017. Genetic variation in CXCR1 haplotypes linked to severity of Streptococcus uberis infection in an experimental challenge model. Vet Immunol Immunopathol 190:45-52.

Song, M., Y. He, H. Zhou, Y. Zhang, X. Li, and Y. Yu. 2016. Combined analysis of DNA methylome and transcriptome reveal novel candidate genes with susceptibility to bovine Staphylococcus aureus subclinical mastitis. Sci Rep 6:29390.

Steeneveld, W., H. Hogeveen, H. W. Barkema, J. van den Broek, and R. B. Huirne. 2008. The influence of cow factors on the incidence of clinical mastitis in dairy cows. Journal of dairy science 91(4):1391-1402.

Suojala, L., L. Kaartinen, and S. Pyorala. 2013. Treatment for bovine Escherichia coli mastitis - an evidence-based approach. J Vet Pharmacol Ther 36(6):521-531.

Taponen, S., E. Liski, A. M. Heikkila, and S. Pyorala. 2017. Factors associated with intramammary infection in dairy cows caused by coagulase-negative staphylococci, Staphylococcus aureus, Streptococcus uberis, Streptococcus dysgalactiae, Corynebacterium bovis, or Escherichia coli. Journal of dairy science 100(1):493-503.

Vangroenweghe, F., L. Duchateau, P. Boutet, P. Lekeux, P. Rainard, M. J. Paape, and C. Burvenich. 2005. Effect of carprofen treatment following experimentally induced Escherichia coli mastitis in primiparous cows. Journal of dairy science 88(7):2361-2376.
Vangroenweghe, F., P. Rainard, M. Paape, L. Duchateau, and C. Burvenich. 2004. Increase of Escherichia coli inoculum doses induces faster innate immune response in primiparous cows. Journal of dairy science 87(12):4132-4144.

Wilson, D. J., Y. T. Grohn, G. J. Bennett, R. N. Gonzalez, Y. H. Schukken, and J. Spatz. 2007. Comparison of J5 vaccinates and controls for incidence, etiologic agent, clinical severity, and survival in the herd following naturally occurring cases of clinical mastitis. Journal of dairy science 90(9):4282-4288.

Ye, R. D. and L. Sun. 2015. Emerging functions of serum amyloid A in inflammation. J Leukoc Biol 98(6):923-929.

Zhang, W., X. Li, T. Xu, M. Ma, Y. Zhang, and M. Q. Gao. 2016. Inflammatory responses of stromal fibroblasts to inflammatory epithelial cells are involved in the pathogenesis of bovine mastitis. Exp Cell Res 349(1):45-52.

**Table 5: Real-Time Quantitative PCR Primer Sequences**

| Gene Name | Primer Sequence (5' → 3') | Reference |
|---|---|---|
| ß-Actin | F: GCAAATGCTTCTAGGCGGACT<br>R: CAATCTCATCTCGTTTTCTGCG | (Pareek et al., 2005) |
| Interleukin-6 | F: TGAGGGAAATCAGGAAAATGT<br>R: CAGTGTTTGTGGCTGGAGTG | (Pareek et al., 2005) |
| Interleukin-8 | F: GCTGGCTGTTGCTCTCTTG<br>R: AGGTGTGGAATGTGTTTTTATGC | (Pareek et al., 2005) |
| Serum Amyloid A3 | F: CCTCAAGGAAGCTGGTCAAG<br>R: TACCTGGTCCCTGGTCATAC | (Bougarn et al., 2011) |
| Tumor Necrosis Factor-α | F: TCTTCTCAAGCCTCAAGTAA<br>R: CCATGAGGGCATTGGCATAC | (Bougarn et al., 2011) |
| Toll-like Receptor-4 [1] | F: AGGCAGCCATAACTTCTCCA<br>R: GCCCTGAAATGTGTCGTCTT | (Sheridan et al., 2016) |
| Toll-like Receptor-4 [2] | F: CCTCTCTAGCCTTCAGGTGC<br>R: TAAAGGCTCTGCACACATCA | Designed in house |

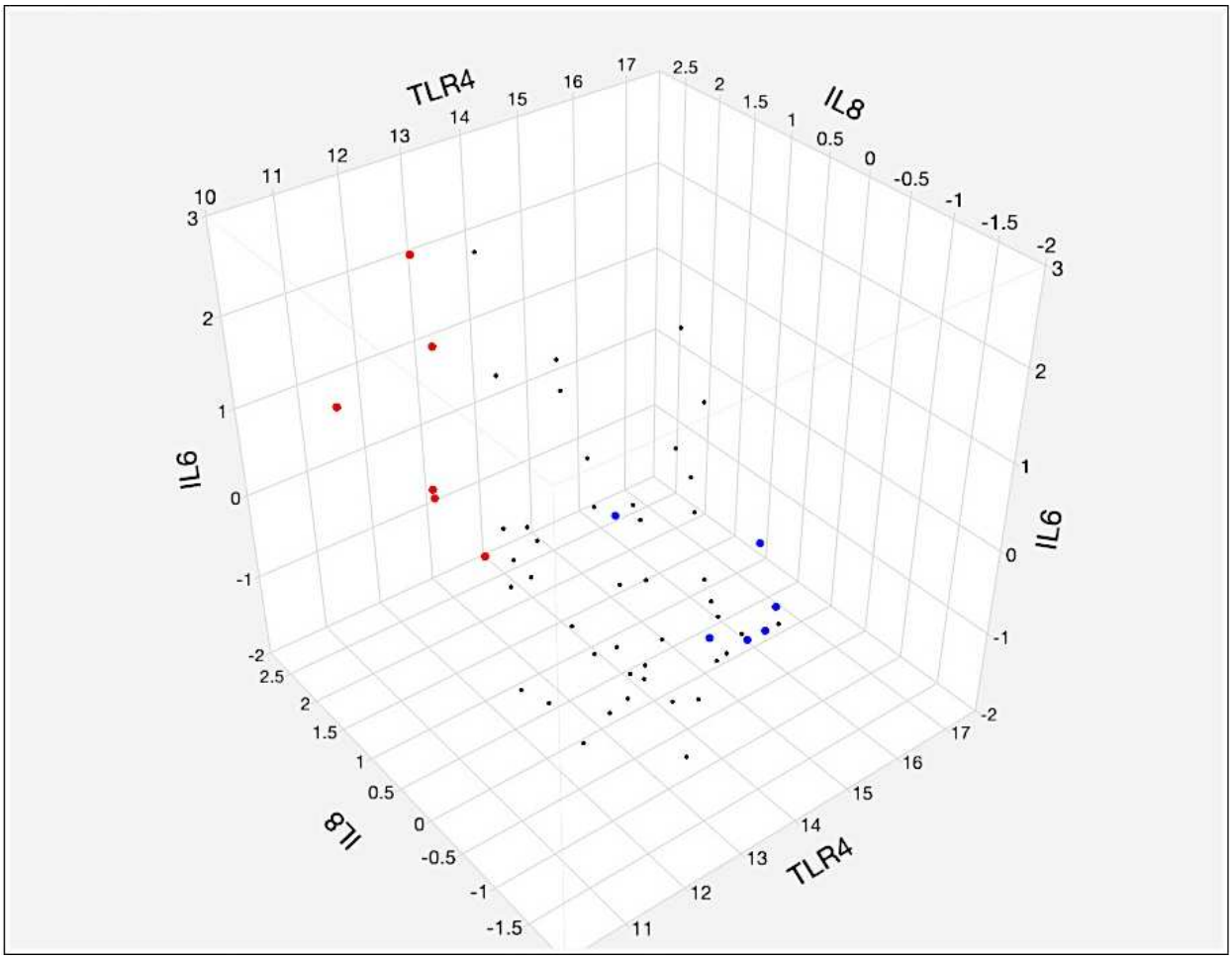


**Figure 3.1: Dermal fibroblast ranking by TLR4 expression and LPS-induced IL-6 and IL-8 production.**

Fibroblasts were revived from cryopreservation and treated with 100 ng/ml of LPS or media alone for 24 hours. Protein levels of IL-8 and IL-6 were measured in challenge media. A normalized rank for LPS response was generated by adjusting IL-8 and IL-6 fg/cell to the challenge batch mean and standard deviation. The resulting variables are presented here as an adjusted rank from -1.50 to 2.33 for IL-8 and from -1.65 to 2.54 for IL-6. Expression of TLR4 was measured in untreated (media only) cultures using two different primer sets, which had a high degree of correlation. Gene expression values are presented as the change in cycles to threshold (ΔCT) of TLR4 versus a housekeeping

gene, β-Actin. The 6 selected high responders are highlighted in red and the 6 selected low responders are highlighted in blue.

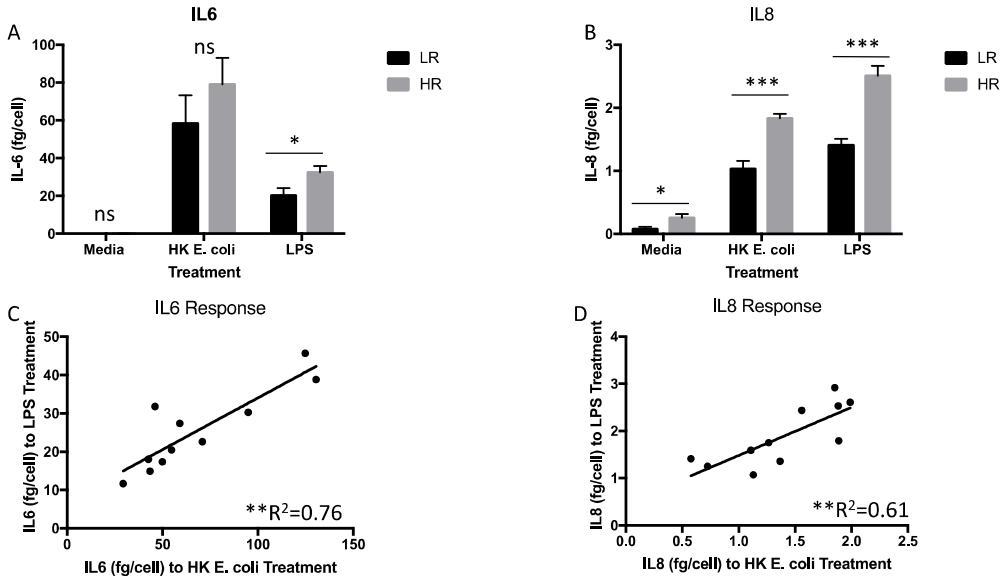


**Figure 3.2: Fibroblast production of IL-6 and IL-8 in response to LPS and heat killed *E. coli*.**

Fibroblasts that were isolated from the 6-selected high (HR) and 6-selected low (LR) responders were revived a second time and challenged side-by-side with heat-killed *E. coli* (MOI 10), 100 ng/ml of LPS or media alone for 24 hrs. Protein concentrations of IL-6 (panel A) and IL-8 (panel B) were then measured in the challenge media and compared using an unpaired Mann-Whitney U test. Results are presented as fg/cell ± SEM and *=P<0.05 and ***=P<0.001. A Spearman rank correlation analysis was performed to compare fibroblast production of IL-6 (panel C) and IL-8 (panel D) when challenged with heat-killed *E. coli* versus LPS and **=P<0.01.

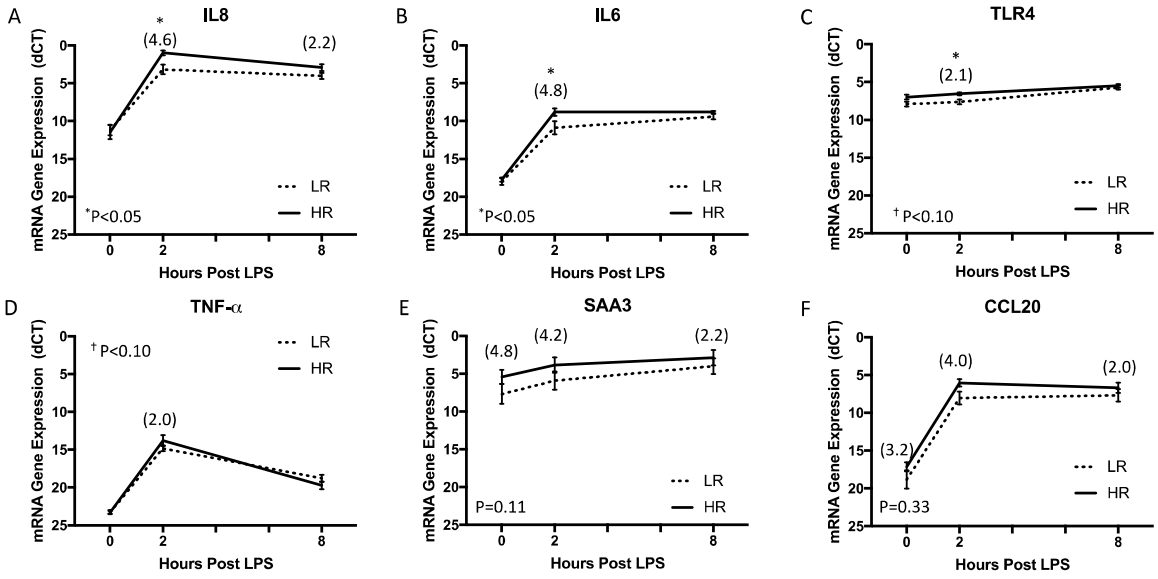


**Figure 3.3. Fibroblast gene expression in response to LPS treatment.**

Fibroblasts from 6 selected high responders (HR) and 6 selected low responders (LR) were treated with 100 ng/ml LPS for 0, 2 and 8 hours. Total RNA was extracted at each time point to determine gene expression levels of (A) IL8, (B) IL6, (C) TLR4, (D) TNF-α, (E) SAA3 and (F) CCL20. Values are presented as the change in cycles to threshold (ΔCT) ± SEM in comparison to β-Actin. The p-values presented in each panel signify the results of a 2-way repeated measures ANOVA that was done to determine the significance of Response x Time gene expression. A Bonferroni's multiple comparisons test was then done to determine significance at each time point where *=P<0.05. Values presented above each time point indicate fold change in gene expression where expression is greater in HR cultures and fold change is ≥2.0.

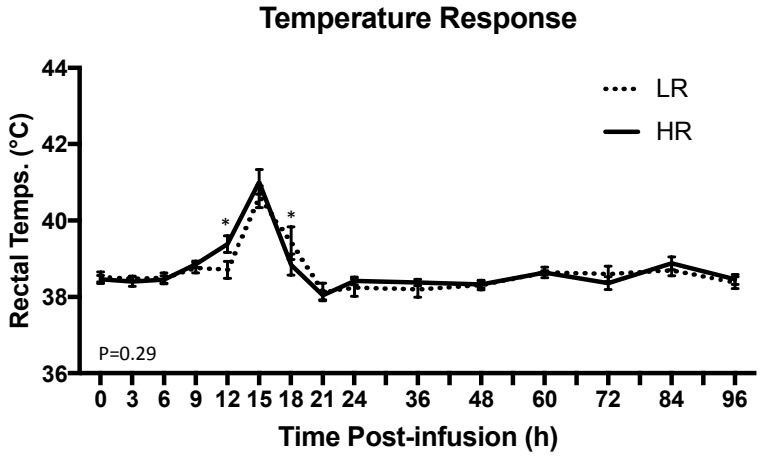


**Figure 3.4: Temperature response.**

Febrile response to *E. coli* infection was measured in high (HR, n=5) and low (LR, n=6) responding animals by monitoring rectal temperatures for 96 hrs. post infection. Temperatures are presented as mean ºC ± SEM. A linear mixed model was used to determine significance based on Response x Time and the corresponding p-value is presented. In addition, multiple comparisons testing was performed using the least square means method to determine significance at each time point where *=P<0.05.

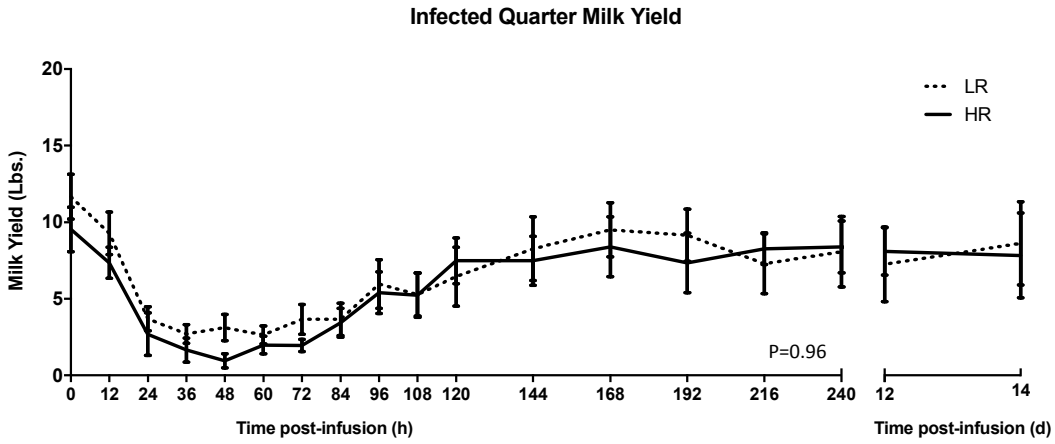


**Figure 3.5: Infected quarter milk production.**

Milk production was measured in the infected right hind quarter for up to 14 days post *E. coli* infection. Milk yield for high (HR, n=5) and low (LR, n=6) responding animals is presented as mean milk weight in lbs. ± SEM. A linear mixed model was used to determine significance based on Response x Time and the corresponding p-value is presented. In addition, multiple comparisons testing was done using the least square means method to determine significance at each time point. There was no significant difference in milk production between HR and LR animals at any time point infection.

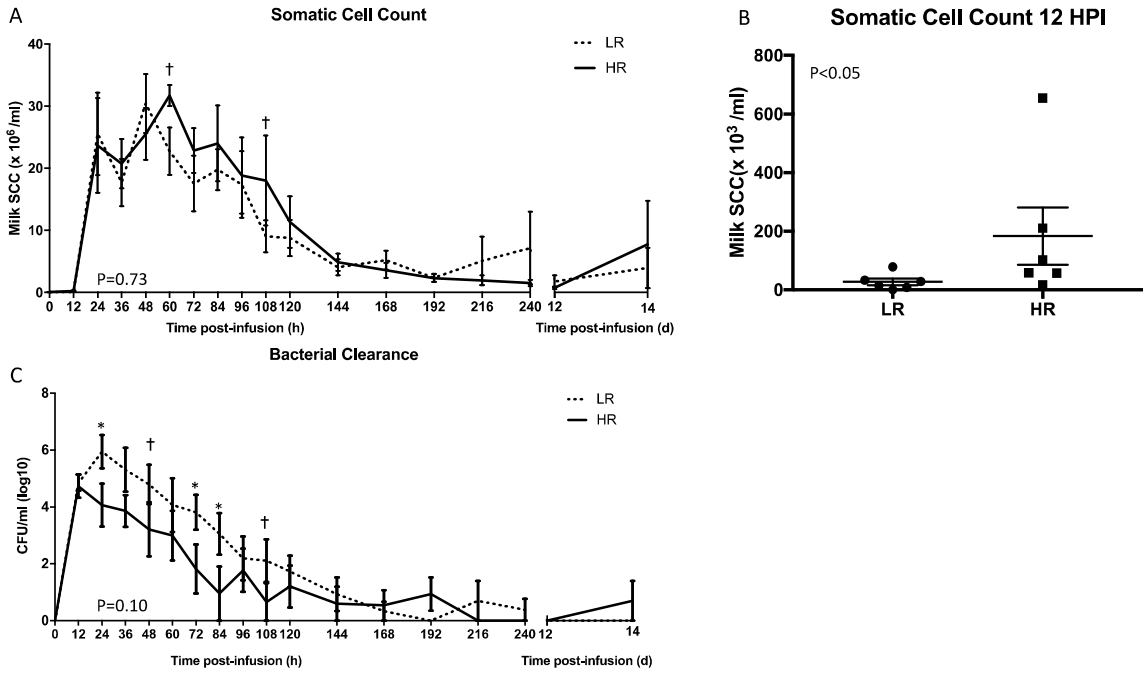


**Figure 3.6: Infected quarter somatic cell count and bacterial clearance.**

Somatic cell count (SCC/ml) and cfu/ml of *E. coli* were measured in milk collected from the infected right hind quarter for up to 14 days post *E. coli* infection. Somatic cell counts for high (HR, n=5) and low (LR, n=6) responding animals are presented as SCC/ml (x $10^6$) (panel A) and SCC/ml (x $10^3$) (panel B) ± SEM. Colony forming units (cfu/ml) of bacteria were log-transformed and are presented as mean cfu/ml ± SEM. A linear mixed model was used to determine significance based on Response x Time for overall SCC and bacteria and corresponding p-values are presented in each panel. In addition, multiple comparisons testing was done using the least square means method to determine significance at each time point where † =P≤0.10 and *=P<0.05. An unpaired Mann-Whitney U test was used to determine significance of SCC increase at 12-hrs. post infection (SCC at 12 HPI – SCC at 0 HPI).

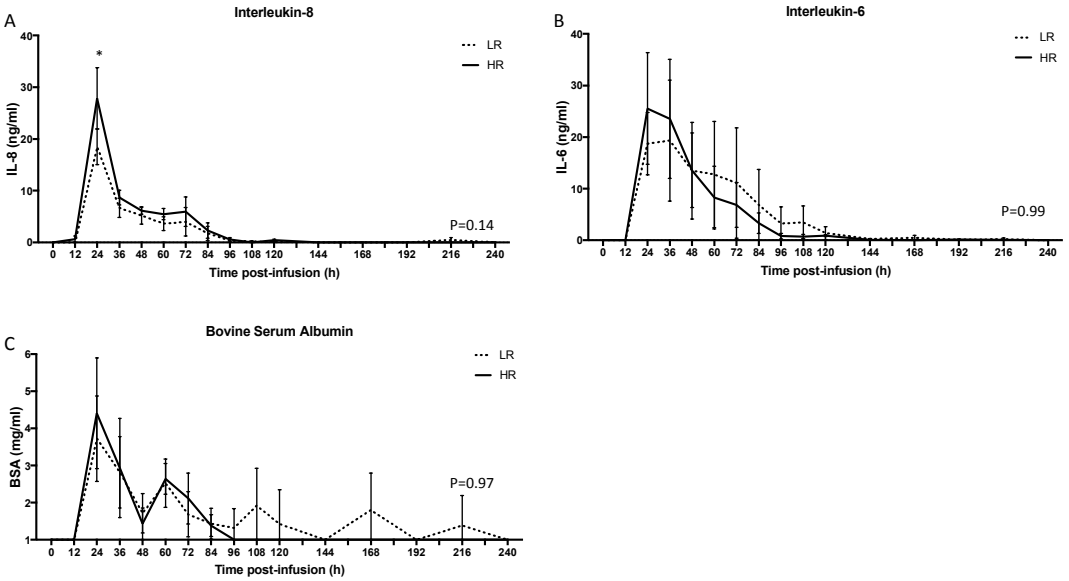


**Figure 3.7: Infected quarter protein response.**

Concentrations of IL-8 (panel A), IL-6 (panel B) and BSA (panel C) were measured in whey collected from the infected right-hind quarter for 240 hours post infection. Levels of IL-8 and IL-6 from high responding (HR, n=5) and low responding (LR, n=6) animals are presented as ng/ml ± SEM. BSA levels from each group are presented as mg/ml ± SEM. A linear mixed model was used to determine significance based on Response x Time and corresponding p-values are presented in each panel. In addition, multiple comparisons testing was performed using the least squares means method to determine significance at each time point and *=P<0.05.

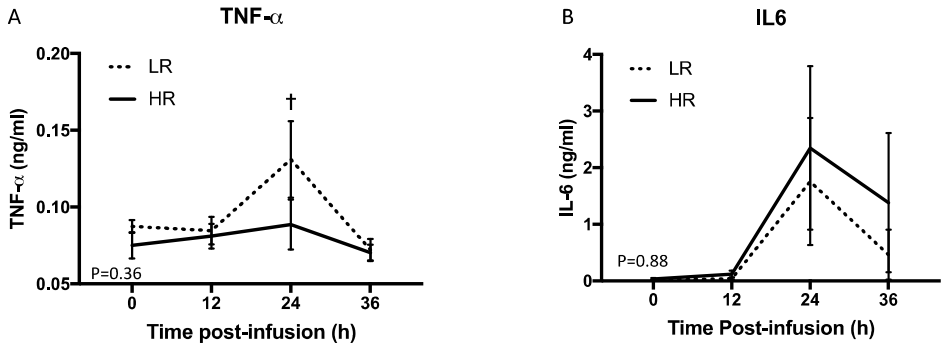


**Figure 3.8: Systemic response to infection.**

Concentrations of circulating TNF-α (panel A) and IL-6 (panel B) were measured for 36 hrs. post infection. Serum cytokine concentrations are presented as mean ng/ml ± SEM and significance was determined using a 2-way repeated measures ANOVA with Bonferroni's multiple comparisons testing to determine significance at each time point where † =P<0.10.

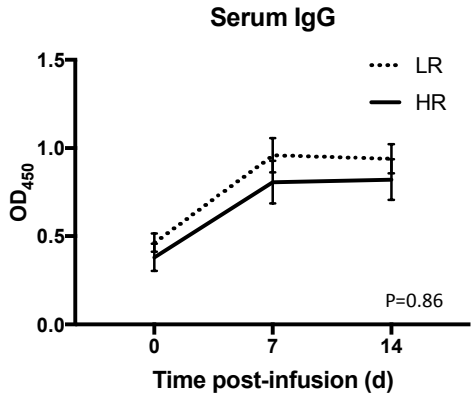


**Figure 3.9: Humoral response to infection.**

*E. coli*-specific total IgG was measured in plasma from high (HR, n=5) and low (LR, n=6) responding animals at days 0, 7 and 14 following E. coli infection. Values are presented as the mean (± SEM) optical density of *E. coli*-specific ELISA wells minus blank control wells. Significance was determined using a 2-way repeated measures ANOVA with Bonferroni's multiple comparisons testing. No significant differences were measured between HR and LR animals at any time post infection.

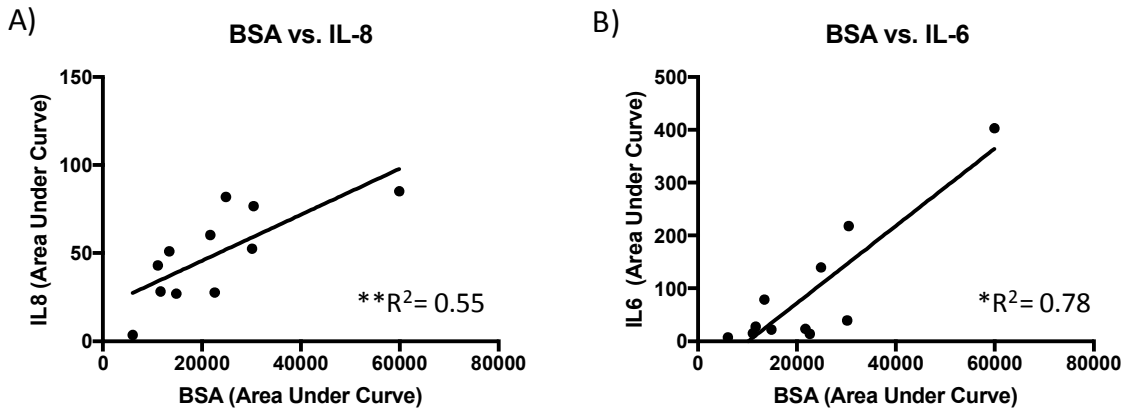


**Figure 3.10: Comparison of milk cytokine response to tissue damage following E. coli infection.**

Area under the curve values of IL8, IL6, and BSA concentrations was determined in milk of all infected animals (5 high responders and 6 low responders) for 10 days post-infection. A Spearman rank correlation analysis was then performed to determine whether milk concentrations of IL-8 (panel A) and IL-6 (panel B) were significantly associated with milk BSA and *=$P<0.05$ and **=$P<0.01$.

# CHAPTER 4: NEONATAL LPS EXPOSURE DOES NOT DIMINISH THE INNATE IMMUNE RESPONSE TO A SUBSEQUENT LPS CHALLENGE IN HOLSTEIN BULL CALVES.

## 4.1. Abstract

The innate immune response following experimental mastitis is quite variable between individual dairy cattle. An inflammatory response that minimizes collateral damage to the mammary gland while still effectively resolving the infection following pathogen exposure is beneficial to dairy producers. The ability of an LPS exposure in early life to generate a low-responding phenotype and thus reduce the inflammatory response to a later life LPS challenge was investigated in neonatal bull calves. Ten Holstein bull calves were randomly assigned to either an early life LPS (ELL) group (n=5) or an early life saline (ELS) group (n=5). At 7 days of age, calves received either LPS or saline, and at 32 days of age all calves were challenged with an intravenous dose of LPS to determine the effect of the early life treatment (LPS or saline) on the immune response generated towards a subsequent LPS challenge. Dermal fibroblast (DF) and monocyte-derived macrophage (MDM) cultures from each calf were established at age 20 and 27 d, respectively, to model sustained impacts from the early life LPS exposure on gene expression and protein production of components within the LPS response pathway. The ELL calves had greater levels of plasma IL-6 and TNF-α than the ELS calves following the early life LPS or saline treatments. However, levels of these two immune markers were similar between ELL and ELS calves when both groups were subsequently challenged with LPS. A comparison of the in vitro LPS-responses of the ELL and ELS

calves revealed similar patterns of protein production and gene expression following an LPS challenge of both DF and MDM cultures established from the treatment groups. While an early life exposure to LPS did not result in a dampened inflammatory response towards a later LPS challenge in these neonatal bull calves, the potential that exposure to inflammation or stress in early life or in utero can create an offspring with a low-responding phenotype as an adult is intriguing and has been documented in rodents. Further work is needed to determine if an inflammatory exposure in utero in a dairy animal would result in a low-responding innate immune phenotype.

Keywords: early life LPS, inter-animal variation, inflammatory response

## 4.2. Introduction

The innate immune system plays a key role in the clearance of pathogens following an infection such as bovine mastitis. This acute, non-specific response is initiated when conserved pathogen associated molecular patterns (PAMP) from a diverse number of pathogens are recognized by germ-line encoded receptors known as pattern recognition receptors (PRR) that are expressed on a variety of cell types. Lipopolysaccharide (LPS) from the cell wall of Gram-negative bacteria, a major causative agent of bovine mastitis, is recognized by the PRR toll-like receptor (TLR) 4. Once LPS is recognized by TLR4, an intracellular signaling cascade activates transcription factors such as nuclear factor-kappa B (NF-κB) that promote the transcription of cytokines including interleukin *(IL) -1β, -6,* and tumor necrosis factor alpha (*TNF-α*) (Kawai and Akira, 2007). These three cytokines play major roles in driving the pro-inflammatory response by promoting febrility, inflammation, and activation of leukocytes to aid in the clearance of the pathogen (Bannerman, 2009).

However, an excessive inflammatory response can be detrimental to the host, causing collateral damage to tissues, such as the mammary gland (Schukken et al., 2011), thereby reducing future milk production and profit for dairy producers (Grohn et al., 2004). The factors that lead to a more robust inflammatory response in some animals and not in others have not been fully elucidated. Even in controlled intra-mammary challenge models of mastitis, inter-animal variation has been observed in cytokine concentrations in milk or blood, milk bacterial counts, and severity of clinical signs (Schukken et al., 1999, Lee et al., 2006, Simojoki et al., 2009), supporting the belief that host factors play a major role in determining the severity and outcome of mammary infections (Burvenich et al., 2003).

Genetic polymorphisms in components of the host's pathogen recognition pathways could lead to the variable immune responses between animals. Within cattle, a number of single nucleotide polymorphisms (SNP) have been identified in genes encoding the toll-like receptors (Novak, 2014), and SNPs in these and other genes involved in the inflammatory response have been associated with individual differences in their resistance to mastitis (Thompson-Crispi et al., 2014). Epigenetic modifications of an animal's DNA can also contribute to the observed phenotypic differences in the immune response of individuals. DNA methylation in particular, has been shown to influence transcription of genes (Moore et al., 2013). In human diseases such as periodontitis and rheumatoid arthritis, aberrant methylation within immune response genes has been linked to an increased risk of disease or severity of signs (Benakanakere et al., 2015, Kojima et al., 2015). The role that epigenetics may play in modulating the bovine innate immune response is now being investigated (Paibomesai et al., 2013, Green

and Kerr, 2014, Chang et al., 2015a) and may provide a more complete picture of how genetic and epigenetic factors combine to produce inflammatory responses following bovine mastitis that range from mild to severe.

While pathogen detection by innate immune cells prompts a robust inflammatory response that is essential to resolving the infection, this response must be under tight regulation to prevent excessive inflammation and extensive tissue damage. There are some mechanisms that can generate a low responding phenotype in an animal, either transiently or long-term, following pathogen exposure. An animal exposed to a low level of LPS can develop a short-term reduction or even an abolished inflammatory response towards a subsequent LPS exposure, thereby exhibiting endotoxin tolerance (Biswas and Lopez-Collazo, 2009). It has been suggested that this tolerized state, generated primarily through macrophages and their reduced production of TNF-α, could be due to a variety of events, including methylation of histone tails, which modify the chromatin structure of anti- and pro-inflammatory genes (El Gazzar et al., 2007), up-regulation of anti-inflammatory or inhibitory genes (Fan and Cook, 2004), and micro-RNA regulation of immune response genes (Baltimore et al., 2008). Additionally, there is abundant evidence that inflammation or disease in early life can have long-term consequences on the innate immune response of an animal. Several studies completed on rodents have shown that neonatal LPS exposure can cause a reduction in the cytokine or febrile response following LPS challenge of the adult rat (Ellis et al., 2005, Spencer et al., 2011). Thus, it would appear that an early life exposure to LPS has the potential to modify the inflammatory response in the adult animal, creating a low-responding phenotype.

In this current study, we investigated if a single exposure to LPS during the neonatal period would modify the in vivo inflammatory response to a subsequent LPS challenge in dairy calves. Additionally, two cellular models, dermal fibroblasts and monocyte-derived macrophages, were utilized to explore if early life exposure to LPS would result in sustained epigenetic modifications of the LPS response of these cells, thereby influencing the innate immune response.

## 4.3. Materials and Methods

### 4.3.1 Experimental design and animal use

Holstein bull calves that were free of clinical disease and had received 4 L of pooled colostrum within 1 h of birth and a second feeding of pooled colostrum 12 h later were purchased from a collaborating dairy farm. The calves (n=10) were brought to the University of Vermont Miller Research Farm at 3.3 ± 0.5 days of age and given a four-day acclimation period. Calves were housed in individual plastic calf hutches and had access to a fenced-in outside area of approximately 3 m x 3 m. Free choice water and a commercially available calf starter were offered to each calf throughout the trial. Calves were bucket-fed an ad libitum amount of a 23% protein, 22% fat milk replacer (OptiMilk Nature's Formula, Arctic Blend; Poulin Grain, Newport, VT) divided between two feedings per day throughout the study period. The University of Vermont Institutional Animal Use and Care Committee approved of all experimental procedures prior to the commencement of this study.

### 4.3.2 Early life treatment

Calves were randomly divided into two groups: Early Life LPS (ELL) and Early Life Saline (ELS), each consisting of five calves. At approximately 7 days of age,

roughly 2 h after the morning feeding, the early life treatment was administered to each calf. Calves in the ELL group received a jugular injection of 0.5 μg of LPS/kg of body weight isolated from *Escherichia coli* O111:B4 (Sigma, St. Louis, MO) diluted in saline, while the calves in the ELS group received an equal volume of saline. The dose of LPS was based on our previous experiments with 14 month-old heifers (Green et al., 2011b). Prior to a single injection of either the LPS solution or saline, the fur along the jugular vein area was clipped and the area was thoroughly cleansed with 70% ethanol. Rectal temperatures were collected immediately prior to the injection (hour 0) and hourly for seven hours. Blood samples were collected at hours 0, 2, and 6 post-LPS infusion with an 18-gauge Vacutainer needle (BD, Franklin Lakes, NJ) and a 10 ml Vacutainer tube containing 158 USP Units of Sodium Heparin (BD). Tubes were inverted several times to ensure adequate mixing of the anti-coagulant and stored on ice for approximately 2 h before centrifugation at 800 x *g* for 20 min at 4°C. Plasma was then collected and stored at -20°C for future analysis.

### 4.3.3 LPS challenge

Each calf was fitted with a jugular catheter for the LPS challenge to allow for easier administration of the LPS solution and blood sampling. Twenty-four hours prior to the catheterization, a 50 cm section of polyethylene tubing (I.D. 1.19mm, O.D. 1.70mm; Intramedic, BD) was cut per calf and sanitized in a Nolvasan S solution (Pfizer, NY, NY). The day of catheterization, each calf was secured in a headlock, the fur was clipped on top of the jugular vein, and the area was cleansed with 70% ethanol. A 12-gauge, 1.5-inch metal hub needle (Hamilton, Reno, NV) was then inserted into the jugular vein, and 20-25 cm of the polyethylene tubing was inserted through the needle. The needle was

then removed, an 18-guage luer stub adapter (Intramedic) attached, and the catheter filled with heparinized saline. The following day, after morning feeding, a single dose of 0.25 μg of LPS/kg of body weight isolated from *E. coli* O111:B4 (Sigma) was infused into each calf at hour 0. Heparinized blood samples were collected at -30 min, and then at hours 1, 2, 3, 5, and 7 post-infusion and stored on ice. Plasma was isolated within 2 h and stored at -20°C for future analysis.

### 4.3.4 Plasma IL-6 and TNF-α quantification

Concentrations of IL-6 in plasma samples were determined with a commercially available bovine IL-6 ELISA kit (Thermo Scientific, Rockford, IL). The capture and detection antibodies were both plated at 1:100 and the streptavidin-horseradish peroxidase was plated at 1:400. Recombinant bovine IL-6 (Thermo Scientific) was used at the assay standard. Concentrations of tumor necrosis factor alpha (TNF-α) were determined via RIA as previously described (Elsasser et al., 2005).

### 4.3.5 Dermal fibroblast isolation

Skin biopsies were collected from the ELL (n=5) and ELS (n=5) calves as previously described (Kandasamy et al., 2011). Briefly, dermal fibroblasts (DF) were isolated following a 6 h digest with 0.5% collagenase type I enzyme (Life Technologies, Grand Island, NY). The collagenase-digested tissue was filtered, centrifuged, and the cell pellet was reconstituted with Dulbecco's modified Eagle medium (DMEM; Hyclone Laboratories, Logan, UT) containing 10% fetal bovine serum (FBS; Hyclone Laboratories), a 1X antibiotic cocktail (100 U/ml of penicillin, 100 μg/ml of streptomycin, and 0.25 μg/ml of amphotericin B; Hyclone Laboratories), and 1X insulin-transferrin-selenium (ITS; Mediatech, Herndon, VA) and seeded into a 25-$cm^2$ flask

(Corning Inc., Corning, NY). Media was replaced after an initial 16 h incubation with fresh DMEM (10% FBS, 1X antibiotic cocktail, and 1X ITS). Once confluency was reached, cells were detached with 0.25% trypsin (MP Biomedical, Santa Ana, CA) and seeded into a 75-$cm^2$ flask. After approximately 4 days, cells were expanded into three 75-$cm^2$ flasks. Finally, confluent cultures were lifted with trypsin and aliquots of the third passage were diluted in DMEM supplemented with 20% FBS and 10% dimethyl sulfoxide (Sigma) and cryopreserved in liquid nitrogen for subsequent revival, expansion, and challenges.

### 4.3.6 Adherent mononuclear cell isolation and culturing

A 180 ml blood sample was obtained from each of the ELL (n=5) and ELS (n=5) calves via jugular venipuncture with a 60 cc syringe pretreated with heparin (10,000 USP units/ml; Sagent Pharmaceuticals, Schaumburg, IL) and immediately transferred to heparinized vacutainer tubes (BD). Samples were stored on ice for approximately one hour prior to processing. Vacutainer tubes were centrifuged at 800 x *g* for 20 min at 4°C. Peripheral blood mononuclear cells (PBMC) were removed and treated with red blood cell lysis buffer (Biolegend, San Diego, CA). No more than five buffy coats were treated with 7.5 ml of 1X red blood cell lysis buffer and inverted for 30 s. Two washes were then performed using sterile Dulbecco's phosphate-buffered-saline (DPBS, Hyclone Laboratories) by centrifuging samples at 500x *g* for 5 min at room temperature. After the second wash, cells were resuspended in 30 ml of DPBS and 10 ml of a Percoll (GE Healthcare, Sweden) solution with a specific density of 1.077 g/$cm^3$ (mixture with 1.5 M NaCl and 30% endotoxin-free water) was carefully underlain below the sample. The Percoll gradient was centrifuged at 400 x *g* for 40 min at room temperature with no

brake. The PBMC layer was removed from the interface of the DPBS and Percoll then placed into a clean polypropylene tube. One more DPBS wash was performed as stated above to remove residual Percoll. After washing, PBMC were resuspended in 30 ml DPBS and centrifuged at 250 x *g* for 12 min at room temperature to remove platelets. After the platelet spin, the cells were resuspended in DMEM, 15% FBS, 1X ITS and 1X penicillin-streptomycin (100 U/ml of penicillin and 100 μg/ml of streptomycin). The PBMC were then transferred to a 25-$cm^2$ flask and incubated in a humidified chamber at 37°C and 5% $CO_2$ for 3 h. Non-adherent cells were removed and placed in a clean polypropylene tube and adherent cells were washed with DPBS and fresh DMEM media was added. Non-adherent cells were transferred to a second 25-$cm^2$ flask and those that had adhered overnight were washed the next morning at which time fresh media was added. Mononuclear cells were incubated for 5 d and media was replaced as needed. After 5 d, cells were lifted with 0.25% trypsin, and the 3 h cultures and overnight cultures were combined and transferred to a 12-well plate at a concentration of 1.0 x $10^5$ cells/ml in a total volume of 1 ml. The cells were cultured for 3 more days to allow for complete macrophage differentiation.

### 4.3.7 In vitro challenges

Aliquots of fibroblast cultures isolated from the ten bull calves were revived in parallel and grown to confluency in a 75-$cm^2$ flask. Cells were then detached with trypsin, counted, and seeded into 6-well plates at a concentration of 1.25 x $10^5$ cells/ml in 2 ml total/well. Following a 24 h incubation, cells were exposed to 500 ng/ml of ultra-pure LPS isolated from *E. coli* O111:B4 (Sigma) or fresh culture media for 24 h. Media was then collected and centrifuged at 10,000x *g* for 1 minute to remove cell debris and

the supernatant was stored at -20°C for future analysis. Cells were lysed at hours 0 and 24 post-LPS, and RNA was extracted at stored at -80°C (5 Prime, Gaithersburg, MD) until future analysis.

After 8 days in culture, macrophages were treated with either media or media containing 100 ng/ml of LPS isolated from *E. coli* O111:B4 for 24 h, after which media was removed and centrifuged at 10,000x *g* for 1 min and supernatant was stored at -20°C until further analysis. Cells were lysed at hours 0 and 24 post-LPS, and RNA was extracted at stored at -80°C (5 Prime, Gaithersburg, MD) until future analysis.

### 4.3.8 Quantification of in vitro immune response proteins

Levels of IL-8 protein produced from fibroblast and MDM cultures following an LPS challenge were determined in duplicate with a commercially available bovine IL-8 ELISA kit (Mabtech, Cincinnati, OH) as per manufacturer's instructions with some modifications. A solution of 1 μg/ml of the monoclonal coating antibody (MT8H6) was plated in 0.05 M bi-carbonate buffer on a high affinity 96-well ELISA plate (Corning Life Sciences, Tewksbury, MA) and incubated overnight at 4°C. Media samples or recombinant bovine IL-8 standard (Thermo Scientific) were plated following a series of three washes with a solution of DPBS and 0.05% Tween-20 (DPBS-T; Fisher Bioreagents, Fair Lawn, NJ) and incubated for 2 h at 20°C. Plates were washed with DPBS-T and a monoclonal detection antibody (26ES-Biotin) was plated at 0.025 μg/ml and incubated for 1 h at 20°C. After a series of washes, streptavidin-horseradish peroxidase (Sigma) was plated at 0.1 μg/ml and incubated for 1 h. Lastly, 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Fisher Scientific, Pittsburgh, PA) was added for approximately 7 min, and the reaction was stopped with 1M $H_2SO_4$.

Concentrations of TNF-α protein in macrophage-conditioned media were determined in duplicate using a custom sandwich ELISA as described above except: coating antibody (polyclonal rabbit anti-bovine TNF-α, 1 mg/ml, Thermo Scientific) was diluted 1:200 in 0.05 M bi-carbonate buffer, recombinant bovine TNF-α (Kingfisher Biotech, Inc., St. Paul, MN) was used as standard, and biotinylated secondary antibody (rabbit anti-bovine TNF-α, Thermo Scientific) was diluted 1:1,000 to a concentration of 0.25 μg/ml in DPBS-T. The detection limit of the assay was 156 pg/ml. The concentrations of IL-1β were similarly quantified using a commercially available sandwich ELISA (Thermo Scientific). The detection limit of the assay was 62 pg/ml. All ELISA reactions were quantitated by measuring absorbance at 450nm and analyzed by a 4-parameter analysis with optical density corrected against blank wells (Synergy-HT, Bio-Tek, Winnoski, VT).

### 4.3.9 Quantitative real time-PCR

Dermal fibroblasts (DF) and monocyte-derived macrophages (MDM) were isolated and cultured for an LPS challenge as described above for each of the ten calves and cell lysate was collected at hours 0 and 24 post-LPS for each culture. The PurefectPure RNA Cultured Cell extraction kit (5 Prime), which includes a DNase treatment step to eliminate DNA contamination, was used to extract total RNA from the cell lysate. RNA concentration was determined using a Qubit Spectrofluorometer (Life Technologies, Carlsbad, CA). The Improm II Reverse Transcriptase Kit (Promega, Madison, WI) was utilized to complete first strand cDNA synthesis. The following genes were selected for real-time PCR analysis: *TLR4, IL6, IL8* (in both DF and MDM cultures) and *CD14, IL-1β receptor*, *and IL-1β* (in MDM cultures only). Expression levels of these

genes were determined by quantitative real-time PCR (qRT-PCR) with a 7500 Fastrun Machine (Applied Biosystems, Carlsbad, CA) using Fermentas Maxima SYBR Green/Fluorescein qPCR Mastermix (Thermo Scientific). An endogenous control gene, *β-actin* was used for normalization of the target genes. Sequences for the constructed primers are listed in Table 1.

### 4.3.10 Statistical analysis

Differences in rectal temperatures, as well as concentrations of plasma TNF-α and IL-6, were determined between the ELL and the ELS calves following the early life treatment and the LPS challenge by a repeated measures ANOVA using Prism 6.0 (GraphPad Software Inc., La Jolla, CA). Differences in immune response proteins and gene expression following an LPS challenge between the fibroblast and monocyte-derived macrophage cultures collected from the ELL and ELS calves were determined by an unpaired t-test in Prism 6.0.

## 4.4. Results

Blood samples were collected from each calf at hours 0, 2, and 6 following the early life treatment (LPS or saline) to determine concentrations of two key inflammatory cytokines, IL-6 and TNF-α. Prior to the treatment, levels of IL-6 (Figure 4.2A) and TNF-α (Figure 4.2B) were similar between the ELL and ELS calves. However, by hour 2 post-treatment, levels of IL-6 and TNF-α in the ELL calves had reached peak values ($P < 0.05$; 14.4 ± 3.1 and 3.2 ± 1.0 ng/ml, respectively), while no increase in either cytokine was observed in the ELS calves. At 6 h post-treatment, IL-6 levels remained elevated ($P < 0.05$) in the ELL calves, and plasma TNF-α had decreased to pre-treatment levels, with no detectable changes in plasma IL-6 or TNF-α from the ELS calves. Moderate

respiratory distress and lethargy was typically observed in the ELL animals. Rectal temperatures did not increase due to the early life treatment in either group and were similar between the ELL and the ELS calves (Figure 4.2C).

Twenty-five days after the early life treatment, an LPS challenge was performed on all ten calves to compare the LPS response between calves that had received LPS (n=5) and calves that had received saline (n=5) in early life. Following an LPS bolus of 0.25 μg/kg of body weight, plasma levels of IL-6 increased in both the ELL and ELS calves, reaching similar peak values (6.7 ± 0.6 and 6.1 ± 0.7 ng/ml) at hour 3, and decreasing slightly by hour 7 post-infusion (Figure 4.3A). Plasma TNF-α reached maximum concentrations in ELL calves 1 h following LPS (4.8 ± 1.3 ng/ml) while TNF-α concentrations peaked in ELS calves 2 h post-LPS (3.9 ± 1.1 ng/ml). Despite the early temporal differences, TNF-α levels were similar between ELL and ELS calves for the remainder of the challenge (Figure 4.3B). Rectal temperatures following the LPS challenge decreased slightly in both groups, as shown in Figure 4.3C, however, there were no differences observed between the ELL and ELS calves.

Skin biopsies were collected from each calf 13 days after the early life treatment and 12 days before the LPS challenge to isolate dermal fibroblasts (DF). This time point was selected in anticipation that any epigenetic modifications resulting from the early life LPS treatment that could influence the fibroblast LPS response would be present in our cellular model. Aliquots of fibroblasts from each animal were revived and challenged in parallel with LPS (500 ng/ml) for 24 h, and levels of IL-6 and IL-8 protein secreted in media were used as indicators of that fibroblast culture's LPS response. Fibroblasts

isolated from the ELL and ELS calves at approximately 20 d of age produced similar levels of IL-6 (Figure 4.4A) and IL-8 (Figure 4.4C) protein following LPS exposure.

Mononuclear cell-derived macrophages (MDM) were isolated from each of the calves (n=10) 21 days after the early life treatment and 4 days before the LPS challenge to determine if the in vitro LPS response from the MDM would be dampened due to the early life LPS treatment. MDM cultures were seeded into 6-well plates, incubated for 24 h, and then challenged with LPS (100 ng/ml) for 24 h. Concentrations of IL-6 and IL-8 protein produced by the MDM cultures following LPS exposure were similar between the treatment groups (Figure 4.4B and 4.4D). Additionally, levels of IL-1β and TNF-α were determined in MDM supernatant following LPS treatment (Figure 4.4E and 4.4F, respectively). Each of the immune response proteins measured in MDM cultures established from the calves were similar between the two early life treatment groups (ELL vs. ELS).

Expression levels of genes involved with the LPS recognition and response pathway were investigated in both the DF and MDM cultures established from all calves. Total RNA was collected from DF and MDM at hours 0 and 24 post-LPS, and gene message was determined by qRT-PCR. Expression of *TLR4*, the receptor responsible for LPS recognition, was consistent following LPS stimulation and was not different between the ELL and ELS groups in either DF or MDM cultures, but was much greater in the MDM cultures (Figure 4.5A and D). The cytokine *IL6* and chemokine *IL8* are critical immune response genes following LPS exposure, and the expression of these genes increased from hour 0 to hour 24 in both cellular models (Figure 4.5B and E). However, no differences were observed between the ELL and ELS calves in either cellular model,

although the expression of both *IL-6* and *IL-8* was much greater in the MDM cultures. Levels of expression of three other genes (*CD14*, *IL-1β receptor*, and *IL-1β)* were determined in control and LPS-treated MDM cultures from the ELL and ELS calves (Figure 4.5G, H, and I). The expression of *IL-1β* was markedly induced by LPS treatment of the MDM cultures, while the expression of *CD14* and the *IL-1β receptor* were not affected. However, there were no differences in gene expression between the cultures established from the ELL and ELS calves.

## 4.5. Discussion

Previous work from our laboratory and others has shown that dairy cattle manifest a wide range of immune responses following experimentally induced *E. coli* (Kornalijnslijper et al., 2003, Bannerman et al., 2004, Kandasamy et al., 2011) or *Staphylococcus aureus* (Schukken et al., 1999, Bannerman et al., 2004, Benjamin et al., 2015a) mastitis. Some animals exhibit a much more robust inflammatory response during mastitis, characterized by high levels of IL-8, IL-6, and TNF-α, which can lead to severe clinical signs and damage to the mammary tissue. Inter-animal variation in the expression of genes within the LPS response pathway, including *TLR4*, the receptor for LPS, may lead to individual differences in the ability to recognize and respond to Gram-negative pathogens (Kandasamy and Kerr, 2012). A genetic component to individual differences is highly likely, but new research suggests that epigenetics may also play a role. For example, we have found that the LPS responsiveness of an animal's fibroblasts changes with age. In side-by-side cultures of cells recovered from cryopreservation but originally collected from the same animals at different ages, we find a marked increase in response to LPS stimulation. Furthermore, the age-dependent difference in LPS response of these

cells can be abolished by treatment with epigenetic modifiers (Green and Kerr, 2014). Expanding on the inter-animal variation observed in the fibroblast model, cultures established from dairy and beef animals displayed contrasting responses following an in vitro LPS exposure. Whole transcriptome analysis of LPS-treated cultures revealed large breed-dependent differences in the expression of many LPS-responsive genes, including *TLR4*, *IL8*, *TNF*, *CCL5,* and *CCL20* (Benjamin et al., 2016b). These genes, which are associated with the inflammatory response to LPS, were expressed between 2.5- and 7.0-fold higher in Holstein fibroblast cultures as compared to Angus cultures. While genetics may contribute to the contrasting in vitro LPS responses between these breeds, offspring from each breed experience very different early life environments, which could lead to varying degrees of epigenetic modifications between the dairy and beef breeds, potentially moderating inflammatory gene expression.

### 4.5.1 Impact of neonatal inflammation

Several studies have shown that inflammation in the neonatal period (shortly after birth) can lead to sustained suppression of the innate immune response to a subsequent inflammatory challenge. Ellis et al. (2005) observed reduced plasma levels of TNF-α and IL-1β as well as a suppressed febrile response following an LPS challenge of adult rats that had been exposed to LPS at 14 days of age compared to saline-exposed controls. Similarly, adult rats that had previously received a neonatal dose of LPS (100 μg/kg) at 14 or 21 days of age produced a lower febrile response compared to neonatal saline controls during a subsequent LPS (50 μg/kg) challenge (Spencer et al., 2006). However, the timing of the neonatal exposure was found to be critical in that LPS treatments given at 7 or 28 days of age were not effective. Additionally, the suppressive effect is not

observed if the adult challenge dose of LPS is increased to a septic dose of 1 mg/kg (Spencer et al., 2010). In the current study, we hypothesized that an early life LPS exposure would generate a low-responding phenotype in Holstein calves towards a subsequent LPS challenge. Surprisingly, ELL calves mounted a similar inflammatory response as the ELS calves during the later LPS challenge that was completed on all calves 25 days after the first LPS or saline exposure. Plasma levels of TNF-α and IL-6 during the later LPS challenge were comparable between treatment groups, showing the early life treatment with LPS (0.5 μg/kg) at 7 days of age did not dampen the subsequent inflammatory response to LPS (0.25 μg/kg). The doses used did produce substantial inflammation and may have been too high to generate the expected result.

Although other studies have shown that administering LPS in the neonatal period does reduce the LPS response in the adult, most of these studies have been completed in rats (Spencer et al., 2011, Wang et al., 2011). Different rates of development and maturity within the immune system between calves and rodents may be one explanation for the lack of differences between our LPS- and saline- treated calves. Offspring from species with a comparatively short gestation period, such as rats (length of 21 days), are born with a less mature immune system than offspring from species with a longer gestation, for example cattle (length of 9 months) (Holsapple et al., 2003). A less mature immune system during the neonatal period in a rat pup may provide an opportunity for inflammation to epigenetically modify the genes of the immune response in the adult, whereas the immune system of a neonatal calf may be less vulnerable to those types of modifications during that same stage of life. Following parturition, the humoral and cell-mediated immune response of the calf is much lower than an adult cow, but within the

first month of life, B-cell numbers increase and the cell-mediated response is similar to that of an adult animal (Barrington and Parish, 2001). The immune system in newborn rats is immature, and continues to develop during the postnatal period and although an immune response can be mounted at 21 days of age, it is not of the same magnitude as an adult animal (Holsapple et al., 2003). A comparison of the degree of immune system development of the rat pups in studies mentioned previously and the calves in this current study suggests the timing of the LPS exposure in the neonatal calves may have been too late to obtain a reduction in the inflammatory response towards an LPS challenge.

### 4.5.2 Fetal programming: maternal inflammation and nutrition

Exposure to bacterial molecules or inflammation in utero can also alter aspects of an offspring's behavior, health, and development. Increases in anxiety and depression-like behaviors, as well as an increased propensity towards developing schizophrenia have been observed in rodents whose dams suffered stress or inflammation during gestation (Ashdown et al., 2006, Depino, 2015). Additionally, several studies have shown that the inflammatory response of the offspring can be suppressed following in utero LPS exposure. For example, Lasala and Zhou (2007) found that an intraperitoneal injection of LPS given to rats on day 18 of pregnancy greatly reduced the inflammatory response (serum levels of TNF-α, IL-1β, and IL-6) of the resulting pups when they were challenged with LPS at 21 days of age. In a similar study, adult rats that were exposed to LPS in utero at days 16, 18, and 20 of gestation have a blunted inflammatory response towards an LPS challenge as compared to saline-exposed rats (Hodyl et al., 2007). Similarly, offspring of mouse dams that were administered LPS approximately 12 h after fertilization had a diminished cytokine response following an LPS challenge at 40 weeks

of age (Williams et al., 2011). Perhaps an in utero exposure of LPS, or maternally derived inflammatory cytokines, would cause a reduction in the inflammatory response towards a later LPS challenge in dairy calves. However, careful consideration of the timing and dosing of the immune stimulant for such a study must be taken, as previous studies conducted in dairy cattle have shown that experimental uterine bacterial infections and LPS infusions can cause abortions prior to gestational term (Miller et al., 1983, Giri et al., 1990).

### 4.5.3 LPS tolerance

Endotoxin tolerance is a protective mechanism that allows for innate immune cells to experience a transient unresponsive period following exposure to low levels of LPS (Biswas and Lopez-Collazo, 2009). Depending on the species, this refractory period can vary in duration, from roughly a week in rats (West and Heagy, 2002), 10 days in dairy cattle (Petzl et al., 2012), to at least two weeks in human patients suffering from endotoxemia (Kox et al., 2011). Several studies have shown that a primary result of endotoxin tolerance is a reduction or complete abolishment of *TNF-α* transcript and protein following a second exposure to LPS (West and Heagy, 2002). Within our study, the calves that were treated with LPS in early life were likely outside the range of a tolerant state during the subsequent LPS challenge that was given 25 days later. The levels of plasma TNF-α in these calves were actually higher during the later LPS challenge compared to levels reached during the LPS treatment in early life (4 ng/ml vs. 3 ng/ml, respectively). This demonstrates that the cells primarily responsible for executing LPS tolerance, monocytes and macrophages (Kox et al., 2011), were able to mount an unabated inflammatory response towards the later LPS challenge.

### 4.5.4 Cellular models of innate immunity

Extensive use of in vitro models to study the bovine innate immune response is evident, with several different cell types, including mammary epithelial cells (Pareek et al., 2005, Gilbert et al., 2013), neutrophils (Sohn et al., 2007, Revelo and Waldron, 2012), and whole blood (Ballou et al., 2015, Jahan et al., 2015) being utilized. However, each of these models has drawbacks, including invasive collection procedures, inability to cryopreserve for subsequent challenges, and a heterogeneous cell population. Conversely, monocyte-derived macrophages do provide a classical innate immune cell model that is relatively easy to collect and culture. Bovine monocyte-derived macrophages have been shown to be responsive to challenges with *Mycobacterium bovis* (Magee et al., 2012) and LPS (Taraktsoglou et al., 2011), resulting in the induction of many immune -response genes such as *IL-8*, *CCL5*, and *TLR4*.

In the current study, the establishment of fibroblast and adherent mononuclear cell cultures from each animal provided two cellular models to determine if the early life LPS induced epigenetic modifications within innate immune response genes would be reflected in the in vitro LPS-response. Additionally, collection of these two cell types from each animal allowed for a comparison of the LPS response of two cellular models from the same animal. Fibroblasts are long-lived cells that are capable of modulating the inflammatory response as they express innate immune receptors such as TLRs and secrete various cytokines and chemokines following pathogen recognition (Buckley et al., 2001, Kandasamy et al., 2011, Benjamin et al., 2015a). Alternatively, circulating monocytes are released from bone marrow, and depending on the environmental conditions, undergo apoptosis within a few days or migrate into tissues (Parihar et al.,

2010). Once inside the tissue, monocytes will differentiate into macrophages that aid in the clearance of a pathogen or damaged cells (Parihar et al., 2010). These tissue-resident macrophages can survive for days to months depending on the needs of the host. Within this study, the main objective in collecting both adherent mononuclear cells and fibroblasts from the saline- and LPS-treated calves was to observe if the early life LPS exposure created a change, either permanent or temporary, in the in vitro LPS response between the early life treatment groups. Monocytes are continually produced in the bone marrow by myeloid progenitor cells, and a systemic cytokine response following LPS exposure may reach these stem cells and induce permanent epigenetic DNA modifications that could be detected in monocytes collected three weeks later. Dermal fibroblasts however are a more stable cell type than monocytes, but may still be susceptible to epigenetic modifications following exposure to a systemic inflammatory response. While epigenetic modifications could be induced within fibroblast progenitor cells, it is more likely that the fibroblast cells we collected three weeks post-challenge were the same cells present at the time of the early life LPS treatment. This may create more transient epigenetic modifications, such as histone modifications, that are still capable of influencing the inflammatory response, but are potentially too short-lived to detect the effect after several rounds of cell division while in cell culture. While progenitor fibroblast cells could be affected, they proliferate at a slower rate, and this effect may not be evident only three weeks post early life treatment. Surprisingly, the in vitro response following an LPS challenge on each of these cell types revealed similar profiles in both cytokine secretion and gene expression between the LPS- and saline-treated calves, which confirmed the lack of differences observed in vivo during the

second LPS challenge. Interestingly, the monocyte-derived macrophages exhibited a much more robust IL-6 response following LPS stimulation as compared to the fibroblasts (Figure 4). Other groups have observed a similar induction of IL-6 in macrophages following LPS stimulation (Jian et al., 1995, Taraktsoglou et al., 2011), but there have been few studies examining differences in the IL-6 response following LPS stimulation between bovine MDM and DF cultures. A comparison between mouse gingival fibroblasts and macrophages revealed that the mouse alveolar macrophage cell line produced less IL-6 following stimulation with LPS compared to the fibroblast cultures (Jones et al., 2010). While this is inconsistent with our findings, Jones et al. (2010) utilized a commercially available cell line from mice and challenged with a LPS dose of 1 μg/ml for 24 h. Our MDM model is derived from primary bovine mononuclear cells that are allowed to differentiate into macrophages in culture, and we exposed the cells to a lower LPS dose (100 ng/ml) for 24 h. Additional challenges that compare the LPS response of primary bovine MDM and DF may be of interest.

### 4.6 Conclusions

Variation in the innate immune response towards mammary infections exists in dairy cattle, with some animals exhibiting a mild inflammatory response and others suffering a severe inflammatory reaction that results in collateral damage or death. It has been shown that exposure to inflammation or stress either during early life or in utero can cause epigenetic modifications to the animal's innate immune response. The potential for this type of exposure to create a low responding phenotype in the offspring, one that mounts an effective inflammatory response without inflicting damage on host's tissue, is exciting. However, based on the results from this study, an early life exposure to LPS

does not cause a dairy calf to mount a milder inflammatory response to a subsequent LPS challenge 25 days later compared to saline-treated control calves. Through the use of mononuclear cell-derived macrophages and dermal fibroblasts, the lack of difference in vivo between LPS-treated and saline-treated calves was confirmed in vitro.

## 4.7 References


Ashdown, H., Y. Dumont, M. Ng, S. Poole, P. Boksa, and G. N. Luheshi. 2006. The role of cytokines in mediating effects of prenatal infection on the fetus: implications for schizophrenia. Molecular psychiatry 11(1):47-55.

Ballou, M. A., D. L. Hanson, C. J. Cobb, B. S. Obeidat, M. D. Sellers, A. R. Pepper-Yowell, J. A. Carroll, T. J. Earleywine, and S. D. Lawhon. 2015. Plane of nutrition influences the performance, innate leukocyte responses, and resistance to an oral *Salmonella enterica* serotype Typhimurium challenge in Jersey calves. Journal of dairy science 98(3):1972-1982.

Baltimore, D., M. P. Boldin, R. M. O'Connell, D. S. Rao, and K. D. Taganov. 2008. MicroRNAs: new regulators of immune cell development and function. Nature immunology 9(8):839-845.

Bannerman, D. D. 2009. Pathogen-dependent induction of cytokines and other soluble inflammatory mediators during intramammary infection of dairy cows. J. Anim. Sci. 87(13 Suppl):10-25.

Bannerman, D. D., M. J. Paape, J. W. Lee, X. Zhao, J. C. Hope, and P. Rainard. 2004. Escherichia coli and Staphylococcus aureus elicit differential innate immune responses following intramammary infection. Clin. Diagn. Lab. Immunol. 11(3):463-472.

Barrington, G. M. and S. M. Parish. 2001. Bovine neonatal immunology. The Veterinary clinics of North America. Food animal practice 17(3):463-476.

Benakanakere, M., M. Abdolhosseini, K. Hosur, L. S. Finoti, and D. F. Kinane. 2015. TLR2 promoter hypermethylation creates innate immune dysbiosis. Journal of dental research 94(1):183-191.

Benjamin, A. L., B. B. Green, B. A. Crooker, S. D. McKay, and D. E. Kerr. 2016. Differential responsiveness of Holstein and Angus dermal fibroblasts to LPS challenge occurs without major differences in the methylome. BMC Genomics 17:in press.

Benjamin, A. L., B. B. Green, L. R. Hayden, J. W. Barlow, and D. E. Kerr. 2015. Cow-to-cow variation in fibroblast response to a toll-like receptor 2/6 agonist and its relation to mastitis caused by intramammary challenge with Staphylococcus aureus. J. Dairy Sci. 98(3):1836-1850.

Biswas, S. K. and E. Lopez-Collazo. 2009. Endotoxin tolerance: new mechanisms, molecules and clinical significance. Trends in immunology 30(10):475-487.

Bougarn, S., P. Cunha, F. B. Gilbert, A. Harmache, G. Foucras, and P. Rainard. 2011. Staphylococcal-associated molecular patterns enhance expression of immune defense genes induced by IL-17 in mammary epithelial cells. Cytokine 56(3):749-759.

Buckley, C. D., D. Pilling, J. M. Lord, A. N. Akbar, D. Scheel-Toellner, and M. Salmon. 2001. Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation. Trends in immunology 22(4):199-204.

Burvenich, C., V. Van Merris, J. Mehrzad, A. Diez-Fraile, and L. Duchateau. 2003. Severity of E. coli mastitis is mainly determined by cow factors. Veterinary research 34(5):521-564.

Chang, G., W. Petzl, J. Vanselow, J. Gunther, X. Shen, and H. M. Seyfert. 2015. Epigenetic mechanisms contribute to enhanced expression of immune response genes in the liver of cows after experimentally induced Escherichia coli mastitis. Vet. J. 203(3):339-341.

Depino, A. M. 2015. Early prenatal exposure to LPS results in anxiety- and depression-related behaviors in adulthood. Neuroscience 299:56-65.

El Gazzar, M., B. K. Yoza, J. Y. Hu, S. L. Cousart, and C. E. McCall. 2007. Epigenetic silencing of tumor necrosis factor alpha during endotoxin tolerance. The Journal of biological chemistry 282(37):26857-26864.

Ellis, S., A. Mouihate, and Q. J. Pittman. 2005. Early life immune challenge alters innate immune responses to lipopolysaccharide: implications for host defense as adults. FASEB J. 19(11):1519-1521.

Elsasser, T. H., J. W. Blum, and S. Kahl. 2005. Characterization of calves exhibiting a novel inheritable TNF-alpha hyperresponsiveness to endotoxin: associations with increased pathophysiological complications. J Appl Physiol (1985) 98(6):2045-2055.

Fan, H. and J. A. Cook. 2004. Molecular mechanisms of endotoxin tolerance. Journal of endotoxin research 10(2):71-84.

Gilbert, F. B., P. Cunha, K. Jensen, E. J. Glass, G. Foucras, C. Robert-Granie, R. Rupp, and P. Rainard. 2013. Differential response of bovine mammary epithelial cells to Staphylococcus aureus or Escherichia coli agonists of the innate immune system. Vet. Res. 44:40.

Giri, S. N., P. Emau, J. S. Cullor, G. H. Stabenfeldt, M. L. Bruss, R. H. Bondurant, and B. I. Osburn. 1990. Effects of endotoxin infusion on circulating levels of eicosanoids, progesterone, cortisol, glucose and lactic acid, and abortion in pregnant cows. Veterinary microbiology 21(3):211-231.

Green, B. B., S. Kandasamy, T. H. Elsasser, and D. E. Kerr. 2011. The use of dermal fibroblasts as a predictive tool of the toll-like receptor 4 response pathway and its development in Holstein heifers. J. Dairy Sci. 94(11):5502-5514.

Green, B. B. and D. E. Kerr. 2014. Epigenetic contribution to individual variation in response to lipopolysaccharide in bovine dermal fibroblasts. Vet. Immunol. Immunopathol. 157(1-2):49-58.

Grohn, Y. T., D. J. Wilson, R. N. Gonzalez, J. A. Hertl, H. Schulte, G. Bennett, and Y. H. Schukken. 2004. Effect of pathogen-specific clinical mastitis on milk yield in dairy cows. J. Dairy Sci. 87(10):3358-3374.

Hodyl, N. A., K. M. Krivanek, E. Lawrence, V. L. Clifton, and D. M. Hodgson. 2007. Prenatal exposure to a pro-inflammatory stimulus causes delays in the development of the innate immune response to LPS in the offspring. J. Neuroimmunol. 190(1-2):61-71.

Holsapple, M. P., L. J. West, and K. S. Landreth. 2003. Species comparison of anatomical and functional immune system development. Birth defects research. Part B, Developmental and reproductive toxicology 68(4):321-334.

Ibeagha-Awemu, E. M., J. W. Lee, A. E. Ibeagha, D. D. Bannerman, M. J. Paape, and X. Zhao. 2008. Bacterial lipopolysaccharide induces increased expression of toll-like receptor (TLR) 4 and downstream TLR signaling molecules in bovine mammary epithelial cells. Vet. Res. 39(2):11.

Jahan, N., A. Minuti, and E. Trevisi. 2015. Assessment of immune response in periparturient dairy cows using ex vivo whole blood stimulation assay with lipopolysaccharides and carrageenan skin test. Veterinary immunology and immunopathology 165(3-4):119-126.

Jian, Z. J., Z. Yang, M. S. Miller, C. D. Carter, D. O. Slauson, and P. N. Bochsler. 1995. Interleukin-6 secretion by bacterial lipopolysaccharide-stimulated bovine alveolar macrophages in vitro. Veterinary immunology and immunopathology 49(1-2):51-60.

Jones, K. J., S. Ekhlassi, D. Montufar-Solis, J. R. Klein, and J. S. Schaefer. 2010. Differential cytokine patterns in mouse macrophages and gingival fibroblasts after stimulation with porphyromonas gingivalis or Escherichia coli lipopolysaccharide. Journal of periodontology 81(12):1850-1857.

Kandasamy, S., B. B. Green, A. L. Benjamin, and D. E. Kerr. 2011. Between-cow variation in dermal fibroblast response to lipopolysaccharide reflected in resolution of inflammation during Escherichia coli mastitis. J. Dairy Sci. 94(12):5963-5975.

Kandasamy, S. and D. E. Kerr. 2012. Genomic analysis of between-cow variation in dermal fibroblast response to lipopolysaccharide. Journal of dairy science 95(7):3852-3864.

Kawai, T. and S. Akira. 2007. Signaling to NF-kappaB by Toll-like receptors. Trends Mol. Med. 13(11):460-469.

Kojima, A., T. Kobayashi, S. Ito, A. Murasawa, K. Nakazono, and H. Yoshie. 2015. Tumor necrosis factor-alpha gene promoter methylation in Japanese adults with chronic periodontitis and rheumatoid arthritis. Journal of periodontal research.

Kornalijnslijper, E., B. Beerda, I. Daemen, J. van der Werf, T. van Werven, T. Niewold, V. Rutten, and E. Noordhuizen-Stassen. 2003. The effect of milk production level on host resistance of dairy cows, as assessed by the severity of experimental Escherichia coli mastitis. Veterinary research 34(6):721-736.
Kox, M., S. de Kleijn, J. C. Pompe, B. P. Ramakers, M. G. Netea, J. G. van der Hoeven, C. W. Hoedemaekers, and P. Pickkers. 2011. Differential ex vivo and in vivo endotoxin tolerance kinetics following human endotoxemia. Crit. Care Med. 39(8):1866-1870.

Lasala, N. and H. Zhou. 2007. Effects of maternal exposure to LPS on the inflammatory response in the offspring. Journal of neuroimmunology 189(1-2):95-101.

Lee, J. W., D. D. Bannerman, M. J. Paape, M. K. Huang, and X. Zhao. 2006. Characterization of cytokine expression in milk somatic cells during intramammary infections with Escherichia coli or Staphylococcus aureus by real-time PCR. Veterinary research 37(2):219-229.

Magee, D. A., M. Taraktsoglou, K. E. Killick, N. C. Nalpas, J. A. Browne, S. D. Park, K. M. Conlon, D. J. Lynn, K. Hokamp, S. V. Gordon, E. Gormley, and D. E. MacHugh. 2012. Global gene expression and systems biology analysis of bovine monocyte-derived macrophages in response to in vitro challenge with Mycobacterium bovis. PLoS ONE 7(2):e32034.

Miller, R. B., S. D. Van Camp, and D. A. Barnum. 1983. The effects of intra-amniotic inoculation of Hemophilus somnus on the bovine fetus and dam. Veterinary pathology 20(5):574-583.

Mills, J. A., D. S. Zarlenga, P. L. Habecker, and R. M. Dyer. 2009. Age, segment, and horn disease affect expression of cytokines, growth factors, and receptors in the epidermis and dermis of the bovine claw. J. Dairy Sci. 92(12):5977-5987.

Moore, L. D., T. Le, and G. Fan. 2013. DNA methylation and its basic function. Neuropsychopharmacology 38(1):23-38.

Novak, K. 2014. Functional polymorphisms in Toll-like receptor genes for innate immunity in farm animals. Veterinary immunology and immunopathology 157(1-2):1-11.

Paibomesai, M., B. Hussey, M. Nino-Soto, and B. A. Mallard. 2013. Effects of parturition and dexamethasone on DNA methylation patterns of IFN-gamma and IL-4 promoters in CD4+ T-lymphocytes of Holstein dairy cows. Canadian journal of veterinary research = Revue canadienne de recherche veterinaire 77(1):54-62.

Pareek, R., O. Wellnitz, R. Van Dorp, J. Burton, and D. Kerr. 2005. Immunorelevant gene expression in LPS-challenged bovine mammary epithelial cells. J Appl Genet 46(2):171-177.

Parihar, A., T. D. Eubank, and A. I. Doseff. 2010. Monocytes and macrophages regulate immunity through dynamic networks of survival and cell death. Journal of innate immunity 2(3):204-215.

Petzl, W., J. Gunther, T. Pfister, C. Sauter-Louis, L. Goetze, S. von Aulock, A. Hafner-Marx, H. J. Schuberth, H. M. Seyfert, and H. Zerbe. 2012. Lipopolysaccharide pretreatment of the udder protects against experimental Escherichia coli mastitis. Innate Immun 18(3):467-477.

Revelo, X. S. and M. R. Waldron. 2012. In vitro effects of Escherichia coli lipopolysaccharide on the function and gene expression of neutrophils isolated from the blood of dairy cows. Journal of dairy science 95(5):2422-2441.

Schukken, Y. H., J. Gunther, J. Fitzpatrick, M. C. Fontaine, L. Goetze, O. Holst, J. Leigh, W. Petzl, H. J. Schuberth, A. Sipka, D. G. Smith, R. Quesnell, J. Watts, R. Yancey, H. Zerbe, A. Gurjar, R. N. Zadoks, H. M. Seyfert, and c. members of the Pfizer mastitis research. 2011. Host-response patterns of intramammary infections in dairy cows. Veterinary immunology and immunopathology 144(3-4):270-289.

Schukken, Y. H., K. E. Leslie, D. A. Barnum, B. A. Mallard, J. H. Lumsden, P. C. Dick, G. H. Vessie, and M. E. Kehrli. 1999. Experimental Staphylococcus aureus intramammary challenge in late lactation dairy cows: quarter and cow effects determining the probability of infection. J. Dairy Sci. 82(11):2393-2401.

Simojoki, H., T. Orro, S. Taponen, and S. Pyorala. 2009. Host response in bovine mastitis experimentally induced with Staphylococcus chromogenes. Veterinary microbiology 134(1-2):95-99.

Sohn, E. J., M. J. Paape, E. E. Connor, D. D. Bannerman, R. H. Fetterer, and R. R. Peters. 2007. Bacterial lipopolysaccharide stimulates bovine neutrophil production of TNF-alpha, IL-1beta, IL-12 and IFN-gamma. Vet. Res. 38(6):809-818.

Spencer, S. J., E. Field, and Q. J. Pittman. 2010. Neonatal programming by neuroimmune challenge: effects on responses and tolerance to septic doses of lipopolysaccharide in adult male and female rats. J. Neuroendocrinol. 22(4):272-281.

Spencer, S. J., M. A. Galic, and Q. J. Pittman. 2011. Neonatal programming of innate immune function. Am. J. Physiol. Endocrinol. Metab. 300(1):E11-18.

Spencer, S. J., S. Martin, A. Mouihate, and Q. J. Pittman. 2006. Early-life immune challenge: defining a critical window for effects on adult responses to immune challenge. Neuropsychopharmacology 31(9):1910-1918.

Taraktsoglou, M., U. Szalabska, D. A. Magee, J. A. Browne, T. Sweeney, E. Gormley, and D. E. MacHugh. 2011. Transcriptional profiling of immune genes in bovine monocyte-derived macrophages exposed to bacterial antigens. Veterinary immunology and immunopathology 140(1-2):130-139.

Thompson-Crispi, K., H. Atalla, F. Miglior, and B. A. Mallard. 2014. Bovine mastitis: frontiers in immunogenetics. Frontiers in immunology 5:493.

Wang, Z. W., P. Wang, F. H. Lin, X. L. Li, X. F. Li, K. T. O'Byrne, S. T. Hou, and R. Y. Zheng. 2011. Early-life exposure to lipopolysaccharide reduces the severity of experimental autoimmune encephalomyelitis in adulthood and correlated with increased urine corticosterone and apoptotic CD4+ T cells. Neuroscience 193:283-290.

West, M. A. and W. Heagy. 2002. Endotoxin tolerance: A review. Critical care medicine 30(1 Supp):S64-S73.

Williams, C. L., J. L. Teeling, V. H. Perry, and T. P. Fleming. 2011. Mouse maternal systemic inflammation at the zygote stage causes blunted cytokine responsiveness in lipopolysaccharide-challenged adult offspring. BMC biology 9:49.

**Table 7: Oligonucleotide primers for expression quantification with real-time PCR**

(The top and bottom sequences are the forward and reverse, respectively)

| Gene Name | Primer (5' to 3') | Reference |
|---|---|---|
| Toll-like receptor 4 | ACTGCAGCTTCAACCGTATC<br>TAAAGGCTCTGCACACATCA | Ibeagha-Awemu et al. (2008) |
| Interleukin - 6 | TGAGGGAAATCAGGAAAATGT<br>CAGTGTTTGTGGCTGGAGTG | Pareek et al. (2005) |
| Interleukin - 8 | GCTGGCTGTTGCTCTCTTG<br>AGGTGTGGAATGTGTTTTTATG | Pareek et al. (2005) |
| Cluster of differentiation 14 | CTCCAGCACCAAAATGAC<br>TCCTCTTCCCTCTCTTCC | Sohn et al. (2007) |
| Interleukin – 1 beta receptor | GCTCGTGTCCTCTCATCACA<br>ACCTTTGTGCTGGTGAATCC | Mills et al. (2009) |
| Interleukin – 1 beta | CTCTCACAGGAAATGAACCGAG<br>GCTGCAGGGTGGGCGTATCACC | Bougarn et al. (2011) |
| B-actin | GCAAATGCTTCTAGGCGGACT<br>CAATCTCATCTCGTTTTCTGCG | Pareek et al. (2005) |

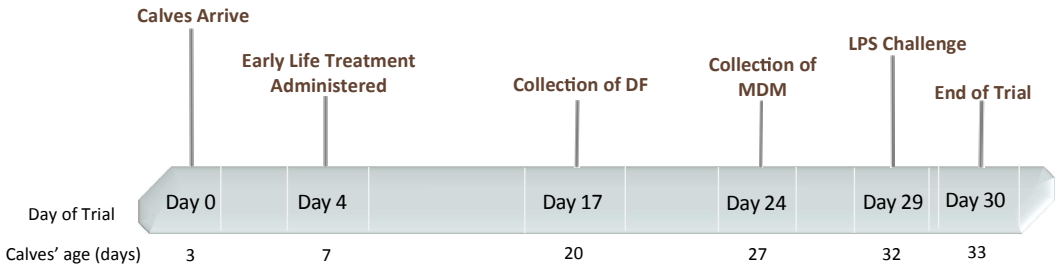


**Figure 4.1: Timeline of the Study**

The effects of an early life LPS exposure on response to a subsequent LPS challenge were determined in ten neonatal Holstein calves. Trial days are shown in the gray bar, with the calf arrival date on day 0 and completion of the trial on day 30, at which point calves were sold. The average age of the calves and key events within the trial are denoted below and above the gray bar, respectively. Early life treatment at day 4 consisted of either an LPS or saline intravenous injection (n=5/treatment). Skin biopsies and blood samples were collected from each calf to isolate dermal fibroblasts (**DF**) and adherent mononuclear cell-derived macrophages (**MDM**), respectively. An LPS challenge was completed on all calves at day 29 of the trial to determine the effect of the early life treatment on response to the subsequent LPS challenge.

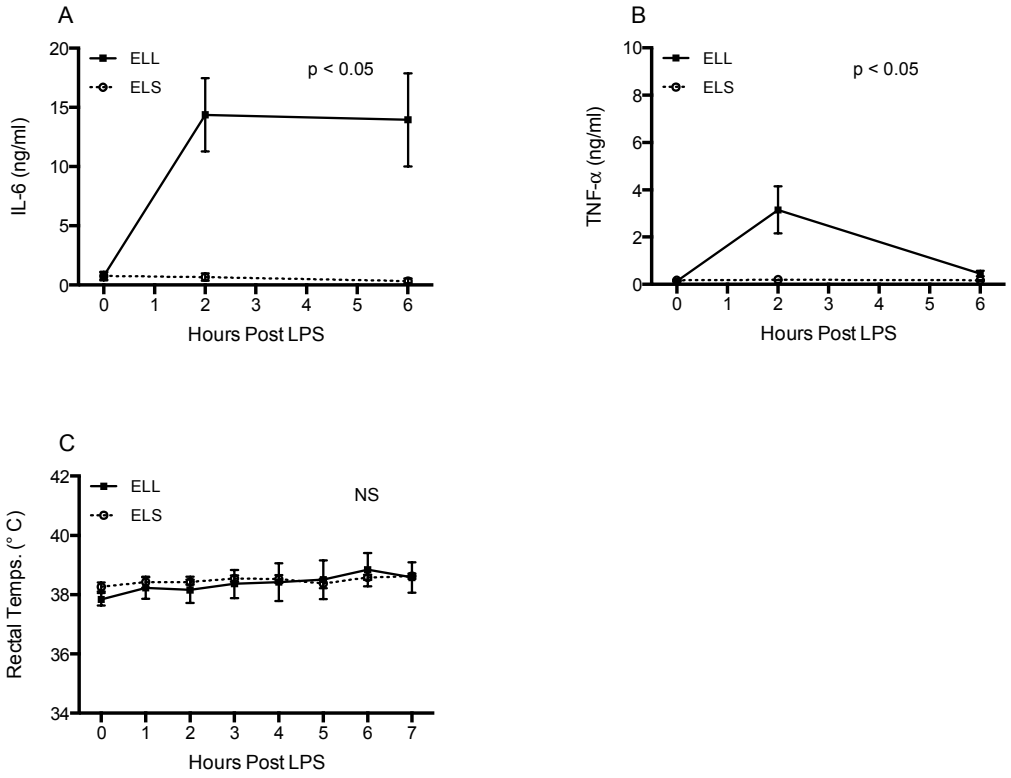


**Figure 4.2: Plasma cytokine responses and rectal temperatures following the early life treatment**

Calves were split into two groups, one of which received LPS and the other received saline at 7 days of age. Blood samples were collected from each calf at hours 0, 2, and 6 following administration of the early life treatment. Plasma levels of IL-6 (A) and TNF- α (B) were determined after the early life treatment, and rectal temperatures were taken on animals from both groups (C). ELL= early life LPS and ELS= early life saline. Values are mean ± SEM.

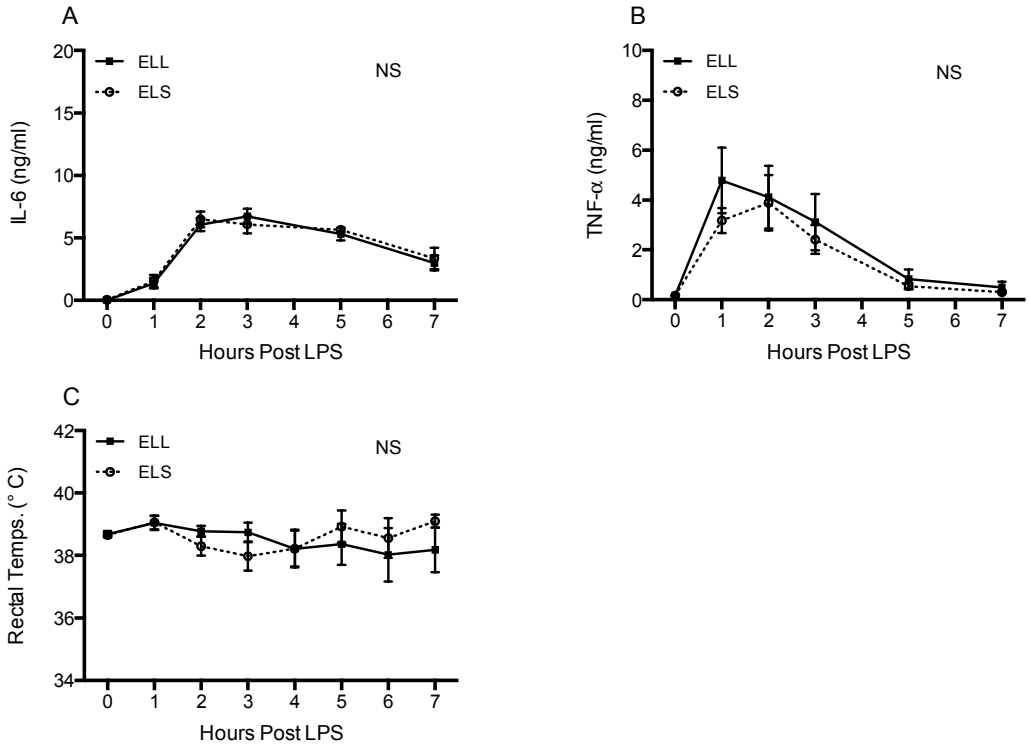


**Figure 4.3: Plasma cytokine response and rectal temperature profiles following an LPS challenge**

Holstein bull calves were divided into two groups (n=5/group) based on an early life treatment consisting of either LPS (ELL) or saline (ELS) that was given at 7 days of age. A subsequent LPS challenge was completed on all calves at 32 days of age to determine if the early life treatment had an effect on the systemic response to the later LPS. Plasma concentrations of IL-6 (A) and TNF- α (B) were measured in the calves from the ELL and ELS groups following the LPS challenge. Rectal temperatures (C) of calves following the LPS intravenous infusion at 32 days of age were not different between the treatment groups. Values are mean ± SEM.

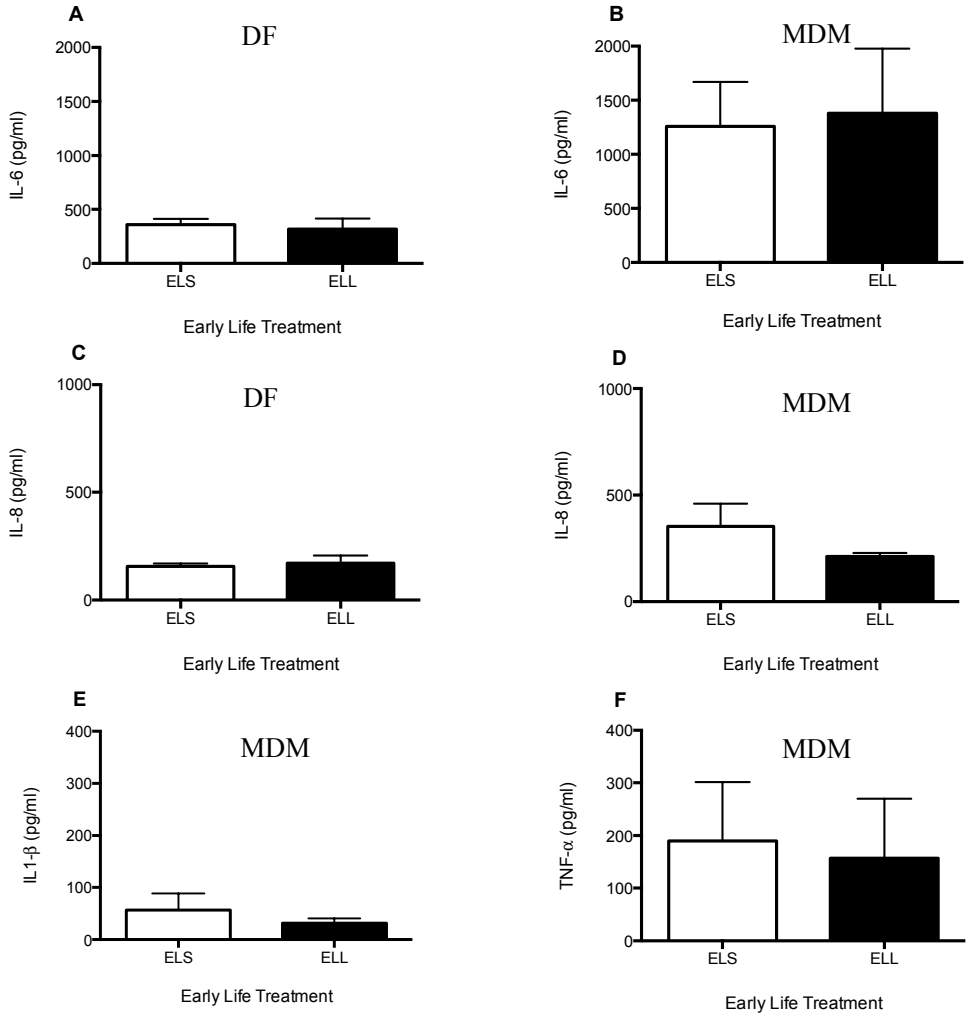


**Figure 4.4: Effect of early life LPS on cellular response to LPS in vitro.**

Dermal fibroblasts (DF) and adherent mononuclear cell-derived macrophages (MDM) were isolated from each calf (n=10) to determine if an in vivo LPS exposure during early life would influence the response of these cell types to an in vitro LPS challenge. A 24 h LPS challenge was completed on each cell type, however DF were treated with 500 ng/ml of LPS and MDM were treated with 100 ng/ml of LPS. The amount of secreted IL-6 from DF (A) and MDM (B), IL-8 from DF (C) and MDM (D) following LPS exposure was determined via an ELISA. Panel (E) and (F) represent the levels of IL-1β and TNF-α

secreted by MDM post-LPS (concentrations of IL-1β and TNF-α were undetectable in LPS-treated fibroblast cultures). ELL= early life LPS, ELS= early life saline. There were no significant differences between treatment groups. Values are mean ± SEM

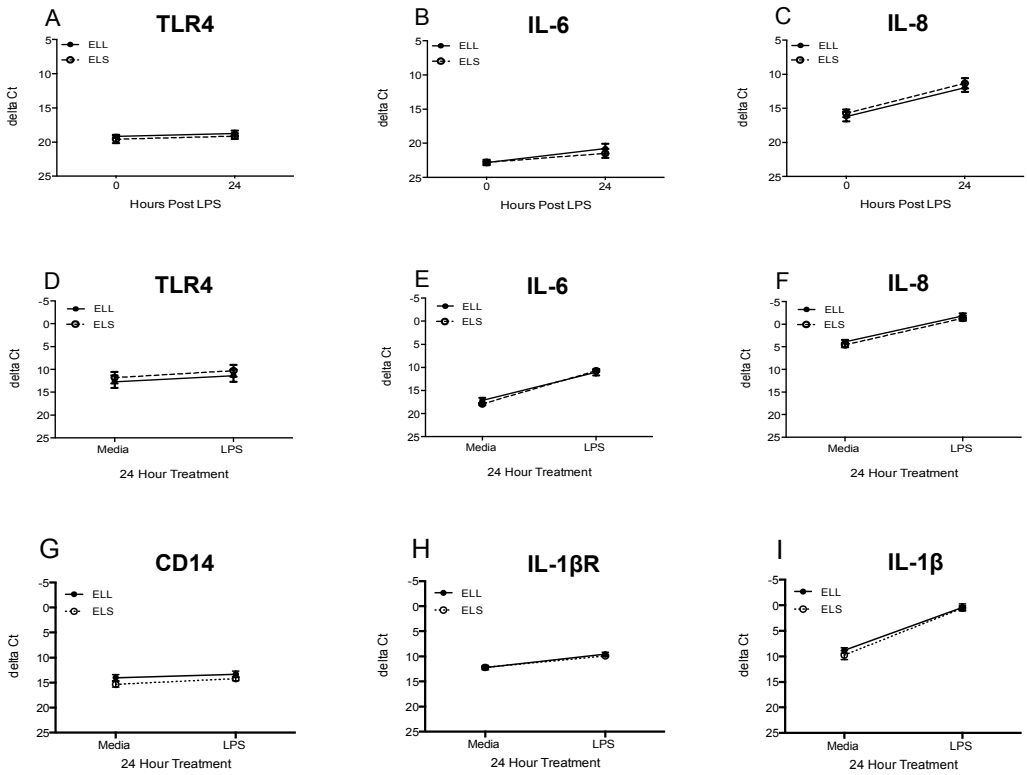


**Figure 4.5: Expression levels of LPS recognition and response genes.**

Dermal fibroblasts (DF) from each calf were revived and challenged with 500 ng/ml of LPS for 24 h and total RNA was collected at hours 0 and 24. A similar challenge was completed on adherent mononuclear cell-derived macrophages (MDM) isolated from each calf; however, an LPS dose of 100 ng/ml was used. Gene expression levels of TLR4, IL-6, and IL-8 post-LPS were investigated on both DF and MDM cultures using qRT-PCR. Expression levels from DF cultures are presented in panels (A) TLR4, (B) IL-6, and (C) IL-8, while expression levels of MDM cultures are in panels (D) TLR4, (E) IL-6, and (F) IL-8. Panels (G), (H), and (I) represent the expression levels of CD14, IL-1β receptor, and IL-1β from MDM, respectively. Expression values for these three genes

were not determined in DF cultures. ELL= early life LPS, ELS= early life saline. There were no significant differences between treatment groups. Values are mean ± SEM.

# CHAPTER 5: GENERAL CONCLUSIONS

Both dairy producers and researchers have struggled with the challenge of effectively controlling bovine mastitis. Although obstacles still remain in the control of contagious pathogens, there has been a significant reduction in cow-to-cow transmission since the implementation of mastitis control plans in the 1960's, specifically the "Five-Point Plan" developed by the National Institute for Research into Dairying (Bramley and Dodd, 1984, Hillerton et al., 1995). Beginning in the 1970's environmental pathogens started gaining attention and have risen to become the most important mastitis-causing pathogens in the modern dairy industry (Oliveira et al., 2013, Ruegg, 2017). Although limited in their duration, coliform bacteria, such as *E. coli*, almost always cause clinical mastitis that is extremely costly to the dairy industry, mainly due to drastic losses in milk production and lowered milk quality. Estimates on the cost of clinical mastitis have ranged from $179 to $444 per episode, depending on age, stage of lactation, cow productivity and parity (Bar et al., 2008, Cha et al., 2011, Rollin et al., 2015, Liang et al., 2017).

There is also substantial between-animal variation in the severity of clinical mastitis that appears to be due to innate differences in host response factors. The contribution of genetic differences to between-cow variation in susceptibility to mastitis has been estimated to be quite low, however the accuracy and magnitude of response to infection that results in differential severity can be addressed through the inclusion of new, more reliable variables in prediction equations. Currently, genetic factors that affect mastitis susceptibility have mainly been linked to infrequent measures of milk somatic cell score which has some inherent flaws. First, somatic cell count is typically taken only

on prescribed monthly basis, without regard to udder health, and will likely miss many transient infections that are characteristic of environmental pathogens or have little value in relation to the dynamic nature of SCC response to infection. Second, a snapshot of somatic cell count shows a very limited picture of the cow's current health status and it may be more valuable to know the duration of elevated somatic cell count and the efficiency of response to recovery. Furthermore, the accurate phenotype has been the subject of considerable debate, whereby some believe that a higher neutrophil response is favorable to infection outcome and others are of the belief that a subdued, but effective response is more desirable to limit tissue damage. In support of the latter supposition, animals ranked as high responders based on fibroblast phenotype that were experimentally infected with *E. coli* had more tissue damage, elevated somatic cell count, and slower return to pre-infection milk production without any benefit in their ability to clear bacteria (Kandasamy et al., 2011).

Although not insubstantial, genetic heritability of mastitis is considered to be quite low, indicating that non-genetic factors may have an equal or greater contribution to mastitis susceptibility. Epigenetic modifications are known to contribute to inter-individual differences in disease susceptibility and severity, including in asthma (Runyon et al., 2012) and *Salmonella enteritidis* infection in chickens (Gou et al., 2012), among many others. In addition, infection status, such as intra-mammary infection with *E. coli* (Chang et al., 2015a) and bovine tuberculosis (Doherty et al., 2016), has been shown to induce epigenetic changes following infection. However, a large gap in knowledge exists in the role of epigenetics in regulating between-animal differences in mastitis severity. Therefore, there is considerable room to improve on producer selection of replacement

calves if epigenetic determinants of mastitis susceptibility are discovered. Selection strategies could be improved using technologies similar to SNP-chips that are currently advancing genetic selection, such that methylation chips in known susceptibility regions could be used to select animals based on epigenetic markers. One concern may be that milk production would suffer as a result of selecting calves with lower risk of mastitis, since increased milk production has been associated with increased risk of mastitis (Rupp and Boichard, 2003). However, knowing the exact genetic and epigenetic factors that account for both reduced severity of mastitis and increased milk production would allow producers to choose animals based on both superior milk production and disease resistance. Furthermore, since epigenetic modifications are closely linked to alterations in environment, it may be possible to treat a calf with a genetic predisposition for high milk production to be more resistant to severe infection.

With these goals in mind, I sought to identify the molecular mechanisms that regulate response to LPS, given that LPS likely reflects the predominant response to *E. coli*. The role of DNA methylation in within-animal variation in response to LPS was first determined with the intent of gaining insight into how between-animal variation in response to *E. coli* is regulated. Dermal fibroblasts isolated from the same animals at 5- and 16-months of age, which exhibit a significant age-related up-regulation of IL-8 and IL-6 response to LPS, were assessed for differences in DNA methylation in unstimulated cultures. Methylation analysis revealed numerous genes that are subject to differential methylation as a result of an increase in fibroblast age and showed that DNA tends to become less methylated with age, a result that correlates with previous studies (Gowers et al., 2011, Marttila et al., 2015). Although we failed to identify any canonical LPS-

response genes that were differentially methylated between the two sets of cultures, numerous innate response genes were hyper-methylated in young cultures, in line with their lower response to LPS. Furthermore, many of the genes hyper-methylated in young cultures have been shown to be regulated by NF-κB and represent potential biomarkers if their relevance can be confirmed with in vivo response to *E. coli*.

Next, with a panel differentially methylated genes established, we wanted to clarify the role of *TLR4* expression and signaling as a potential contributor to mastitis severity. As such, fibroblasts were isolated from a cohort of sixty adult, lactating animals and differences in baseline *TLR4* expression and LPS-induced IL-8 and IL-6 protein production were measured. Animals were subsequently ranked from low to high based on the three variables, and phenotypes of high and low responders were confirmed. The impact of *TLR4* expression and signaling on severity of mastitis was then determined by challenging six high- and six low-responders with an intra-mammary *E. coli* infection. Interestingly, high responders had an earlier elevation in somatic cell count and fever response that corresponded with their ability to clear bacteria more efficiently from the infected gland. However, significant between-animal variation in response was still evident, and mammary gland recovery, as measured by tissue damage and milk production, was highly variable and similar between high and low responders. These results suggest that while rapid-responding animals are able to clear bacteria more effectively, down-regulation of response and tissue recovery does not differ between high and low responders, and likely requires the identification of additional parameters within the resolution phase of infection. Furthermore, results from Chapter 3 contradict earlier research within the lab that first assessed whether fibroblast responsiveness, based on

LPS-induced IL-8 production, could predict mastitis severity. In this study, animals whose fibroblasts produce more IL-8 in response to LPS exhibit increased tissue damage and lower milk production, but lack any difference in their ability to clear bacteria in comparison to animals with lower responding fibroblasts (Kandasamy et al., 2011).

In light of this discrepancy, I re-analyzed the animals described in chapter 3, in addition to a second set of 12 animals that were infected with *E. coli* in an identical pilot study. Using milk production recovery at 14-days post-infection as a marker for mastitis severity, the five animals with the most severe mastitis and the five animals with the most mild mastitis were identified and compared. As shown in figure 5.1, there is a significant variation between-animals in their ability to return to pre-infection milk production that is concomitant with large differences in the duration of their neutrophil response. Although neutrophil influx is identical between the two groups of animals until 48-hrs. post-infection, severe animals maintain an elevated response. Milk IL-8 is similarly sustained in severe animals and consequently, severe animals have dramatically higher amounts of mammary gland tissue damage, as measured by milk BSA (figure 5.2).

Interestingly, while it was anticipated that high and low responders would not exhibit differences in their ability to clear bacteria, animals ranked as severe responders have a much higher bacterial burden (figure 5.3). This led to the question of whether initial leukocyte levels differed between animals with varying abilities to recover from infection. A Pearson correlation analysis comparing the ratio of baseline somatic cell count to bacterial dose and milk production at 10 days post-infection in 32 animals infected with *E. coli* (24 from the previous analysis and 8 from the original 2011 study) showed no correlation of initial leukocyte levels and mastitis severity (figure 5.4). At 14

days post-infection, there is a small, but statistically significant correlation between initial SCC to bacteria ratio (24 animals only, figure 5.4) and mastitis severity, however the biological relevance of this association should be confirmed in a much larger cohort of animals. Perhaps just as important is the effectiveness of leukocyte response, which may be lessened in animals with severe mastitis, as has been shown with patients that become septic in response to Gram-negative infection (Rinchai et al., 2012). Future studies should assess other cell types, perhaps in conjunction with fibroblasts, to gain a more accurate picture of mammary gland response.

Finally, to determine whether early life endotoxin would result in a more subdued response to a subsequent LPS treatment as was hypothesized, neonatal bull calves were treated intravenously with either LPS or saline at one-week of age. Following early life treatment, cellular changes were measured in two different cell types, primary dermal fibroblasts and monocyte-derived-macrophages. Unfortunately, no differences in LPS response were measured in fibroblast cultures and surprisingly, we also failed to detect any changes in MDM response, although literature suggests that they are amenable to epigenetic modulation following exposure to endotoxin. The second intravenous LPS challenge also confirmed the lack of difference in cellular response in that calves treated with early life LPS responded similarly to saline treated calves.

Although much more work is still required, our results lend significant insight into the epigenetic regulation of the bovine innate immune response to LPS and how differences in *TLR4* expression and signaling contributes to mastitis severity. Furthermore, this is the first study to determine the effects of neonatal LPS exposure on calf innate immunity. While considerable research has focused on the bovine innate

immune response to mastitis-causing pathogens, it has been mostly limited to the ideal cellular and cytokine response to pathogens. Conversely, there is significantly less known about how the mammary gland recovers from the assault of both bacteria and leukocytes, and as our results have shown, this may prove to be the missing link into our understanding of severe mastitis susceptibility. In addition, as has already been established in rodent models, it will be necessary to define the crucial developmental time frame when epigenetic modifications exhibit the most plasticity to environmental stimuli before early-life interventions can be used to modulate immunity. To conclude, the current work is one of the first to integrate the collective impact of DNA methylation, differential regulation of TLR4 signaling and neonatal environmental exposures on dairy cow response to *E. coli* mastitis. Our results show that there is still significant room to improve on reducing severe mastitis susceptibility based on the collaborative effect of both environment and epigenetics on genetic predisposition.

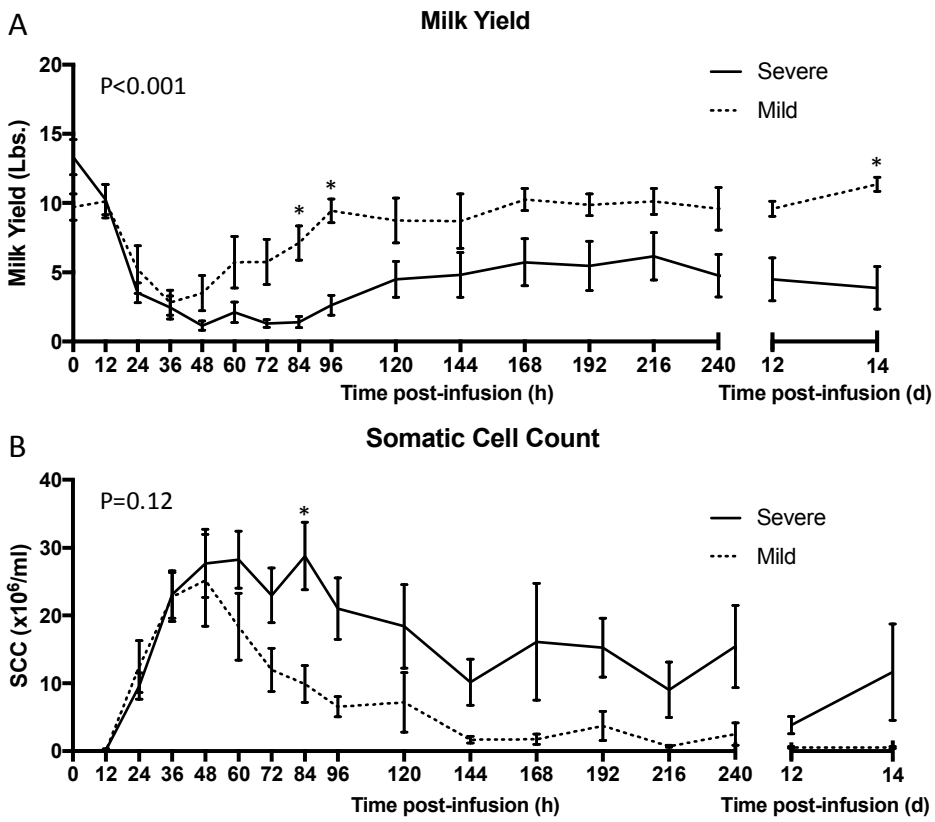


**Figure 5.1: Infected quarter milk yield and somatic cell count in animals with severe and mild mastitis.** Milk production was measured in the infected quarter of 24 animals experimentally treated with 200 cfu of *E. coli* in two separate challenges. Animals with the highest (Mild, n=5) and lowest (Severe, n=5) recovery at 14-days post infection (milk yield day 14 – milk yield day 0) are summarized in panel A and mean milk weight is presented in lbs. ± SEM. Somatic cell count (SCC/ml) was also measured in the infected quarters of severe and mild animals and is presented as SCC/ml (x $10^6$) ± SEM in panel B. P-values presented in each panel were measured using a 2-way repeated measures ANOVA with Bonferroni's multiple comparison testing to determine Phenotype X Time significance where *=P<0.05 at each time point.

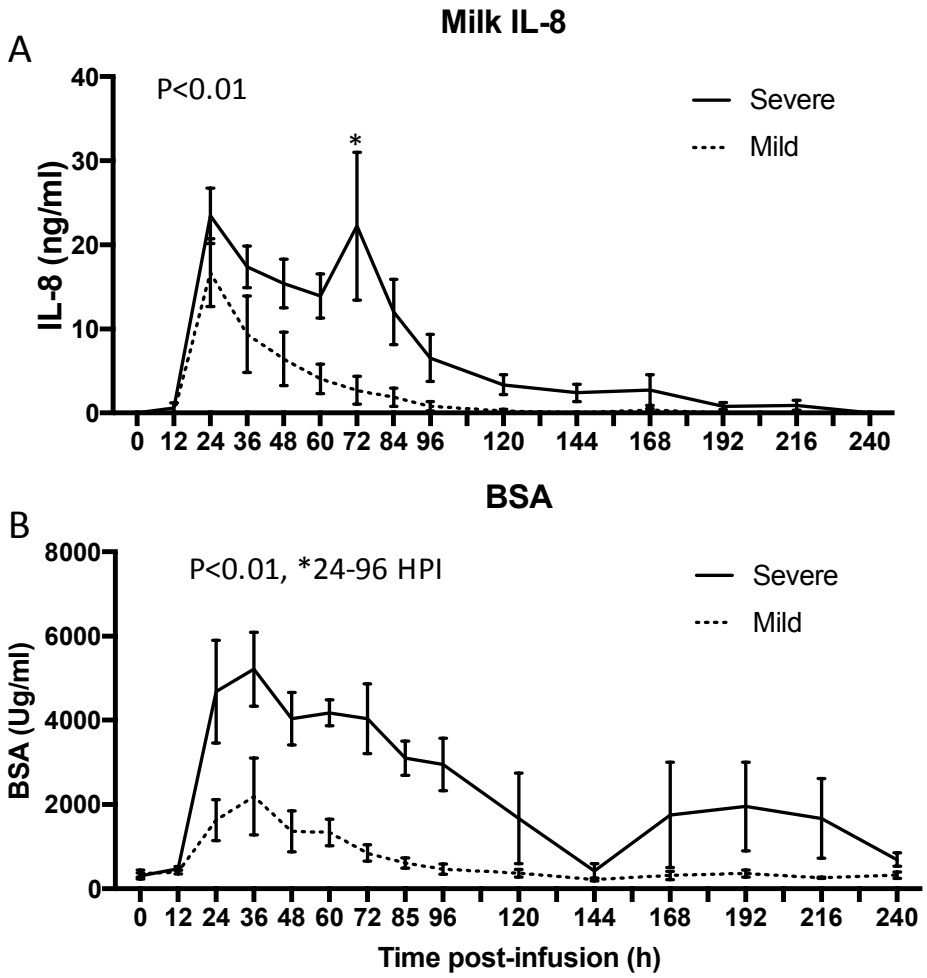


**Figure 5.2: Infected quarter IL-8 and bovine serum albumin in animals with severe and mild mastitis.** Milk IL-8 and BSA were measured in the *E. coli* infected quarter of 10 animals with phenotypic differences in mastitis severity (Mild, n=5) (Severe, n=5). Interleukin-8 concentrations are summarized in panel A and presented as ng/mL ± SEM. Bovine serum albumin concentrations are summarized in panel B and presented as Ug/mL ± SEM. P-values presented in each panel were determined using a 2-way repeated measures ANOVA with Bonferroni's multiple comparison testing to determine Phenotype X Time significance where *=P<0.05 at each time point.

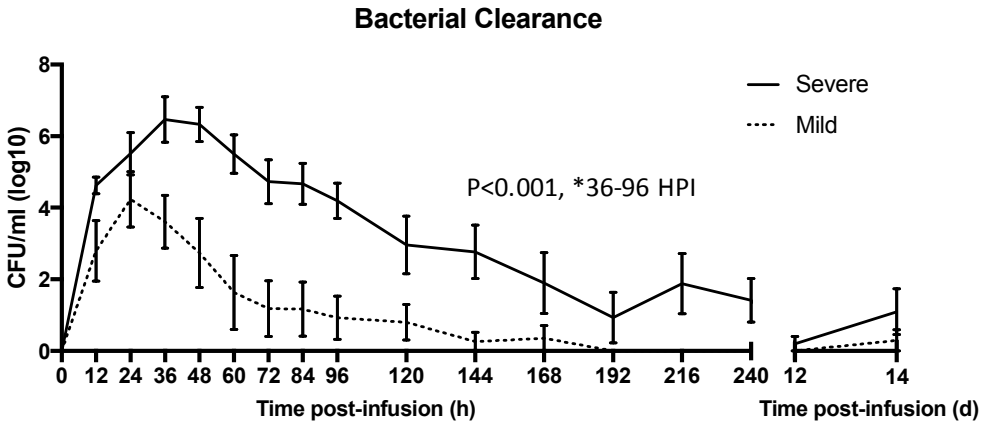


**Figure 5.3: Infected quarter bacterial recovery in animals with severe and mild mastitis.** Recovery of bacteria was measured in the *E. coli* infected quarter of 10 animals with phenotypic differences in mastitis severity (Mild, n=5) (Severe, n=5) and is presented as log-transformed CFU/mL ± SEM. P-values presented above were determined using a 2-way repeated measures ANOVA with Bonferroni's multiple comparison testing to determine Phenotype X Time significance where *=P<0.05 at each time point.

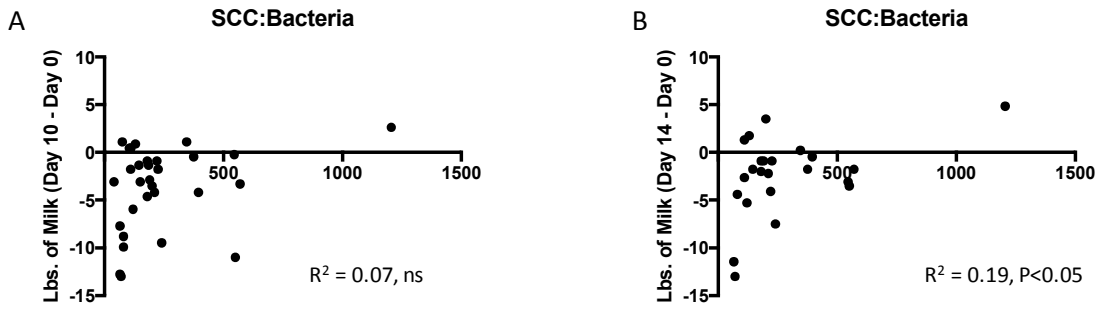


**Figure 5.4: Correlation of baseline SCC to bacteria ratio and milk yield recovery at day 10 and day 14.** The ratio of baseline (0 h) somatic cell count (SCC/ml) and initial inoculum (200 cfu *E. coli*) was calculated and compared to milk yield recovery (lbs) at day 10 (panel A, n=32) and day 14 (panel B, n=24). A Pearson correlation analysis was then conducted to determine whether baseline ratio of SCC to bacteria was associated with a difference in ability to recover to pre-infection milk production. Correlation as measured by R-squared values and significance are displayed in their corresponding panels.

## COMPREHENSIVE BIBLIOGRAPHY

Aitken, S. L., C. M. Corl, and L. M. Sordillo. 2011. Immunopathology of mastitis: insights into disease recognition and resolution. J Mammary Gland Biol Neoplasia 16(4):291-304.

Alain, K., N. A. Karrow, C. Thibault, J. St-Pierre, M. Lessard, and N. Bissonnette. 2009. Osteopontin: an early innate immune marker of Escherichia coli mastitis harbors genetic polymorphisms with possible links with resistance to mastitis. BMC Genomics 10:444.

Alexaki, V. I., V. Pelekanou, G. Notas, M. Venihaki, M. Kampa, V. Dessirier, S. Sabour-Alaoui, E. N. Stathopoulos, A. Tsapis, and E. Castanas. 2012. B-cell maturation antigen (BCMA) activation exerts specific proinflammatory effects in normal human keratinocytes and is preferentially expressed in inflammatory skin pathologies. Endocrinology 153(2):739-749.

Alfonseca-Silva, E., R. Hernandez-Pando, and J. A. Gutierrez-Pabello. 2016. Mycobacterium bovis-infected macrophages from resistant and susceptible cattle exhibited a differential pro-inflammatory gene expression profile depending on strain virulence. Vet Immunol Immunopathol 176:34-43.

Andersen-Nissen, E., T. R. Hawn, K. D. Smith, A. Nachman, A. E. Lampano, S. Uematsu, S. Akira, and A. Aderem. 2007. Cutting edge: Tlr5-/- mice are more susceptible to Escherichia coli urinary tract infection. J Immunol 178(8):4717-4720.

Ashdown, H., Y. Dumont, M. Ng, S. Poole, P. Boksa, and G. N. Luheshi. 2006. The role of cytokines in mediating effects of prenatal infection on the fetus: implications for schizophrenia. Molecular psychiatry 11(1):47-55.

Bacalini, M. G., S. Friso, F. Olivieri, C. Pirazzini, C. Giuliani, M. Capri, A. Santoro, C. Franceschi, and P. Garagnani. 2014. Present and future of anti-ageing epigenetic diets. Mech Ageing Dev 136-137:101-115.

Ballou, M. A. 2012. Growth and Development Symposium: Inflammation: Role in the etiology and pathophysiology of clinical mastitis in dairy cows. Journal of animal science 90(5):1466-1478.

Ballou, M. A., D. L. Hanson, C. J. Cobb, B. S. Obeidat, M. D. Sellers, A. R. Pepper-Yowell, J. A. Carroll, T. J. Earleywine, and S. D. Lawhon. 2015. Plane of nutrition influences the performance, innate leukocyte responses, and resistance to an oral Salmonella enterica serotype Typhimurium challenge in Jersey calves. Journal of dairy science 98(3):1972-1982.

Baltimore, D., M. P. Boldin, R. M. O'Connell, D. S. Rao, and K. D. Taganov. 2008. MicroRNAs: new regulators of immune cell development and function. Nature immunology 9(8):839-845.

Bannerman, D. D. 2009. Pathogen-dependent induction of cytokines and other soluble inflammatory mediators during intramammary infection of dairy cows. J. Anim. Sci. 87(13 Suppl):10-25.

Bannerman, D. D., A. C. Kauf, M. J. Paape, H. R. Springer, and J. P. Goff. 2008. Comparison of Holstein and Jersey innate immune responses to Escherichia coli intramammary infection. Journal of dairy science 91(6):2225-2235.

Bannerman, D. D., M. J. Paape, J. W. Lee, X. Zhao, J. C. Hope, and P. Rainard. 2004. Escherichia coli and Staphylococcus aureus elicit differential innate immune responses following intramammary infection. Clin. Diagn. Lab. Immunol. 11(3):463-472.

Bar, D., L. W. Tauer, G. Bennett, R. N. Gonzalez, J. A. Hertl, Y. H. Schukken, H. F. Schulte, F. L. Welcome, and Y. T. Grohn. 2008. The cost of generic clinical mastitis in dairy cows as estimated by using dynamic programming. Journal of dairy science 91(6):2205-2214.

Barra, M. M., D. M. Richards, J. Hansson, A. C. Hofer, M. Delacher, J. Hettinger, J. Krijgsveld, and M. Feuerer. 2015. Transcription Factor 7 Limits Regulatory T Cell Generation in the Thymus. J Immunol 195(7):3058-3070.

Barrington, G. M. and S. M. Parish. 2001. Bovine neonatal immunology. The Veterinary clinics of North America. Food animal practice 17(3):463-476.

Baylis, D., D. B. Bartlett, H. P. Patel, and H. C. Roberts. 2013. Understanding how we age: insights into inflammaging. Longev Healthspan 2(1):8.

Begue, G., U. Raue, B. Jemiolo, and S. W. Trappe. 2017. DNA Methylation Assessment from Human Slow- and Fast-Twitch Skeletal Muscle Fibers. J Appl Physiol (1985):jap 00867 02016.

Benakanakere, M., M. Abdolhosseini, K. Hosur, L. S. Finoti, and D. F. Kinane. 2015. TLR2 promoter hypermethylation creates innate immune dysbiosis. Journal of dental research 94(1):183-191.

Benjamin, A. L., B. B. Green, B. A. Crooker, S. D. McKay, and D. E. Kerr. 2016a. Differential responsiveness of Holstein and Angus dermal fibroblasts to LPS challenge occurs without major differences in the methylome. BMC Genomics 17(1):258.

Benjamin, A. L., B. B. Green, B. A. Crooker, S. D. McKay, and D. E. Kerr. 2016b. Differential responsiveness of Holstein and Angus dermal fibroblasts to LPS challenge occurs without major differences in the methylome. BMC Genomics 17:in press.

Benjamin, A. L., B. B. Green, L. R. Hayden, J. W. Barlow, and D. E. Kerr. 2015a. Cow-to-cow variation in fibroblast response to a toll-like receptor 2/6 agonist and its relation to mastitis caused by intramammary challenge with Staphylococcus aureus. J. Dairy Sci. 98(3):1836-1850.

Benjamin, A. L., B. B. Green, L. R. Hayden, J. W. Barlow, and D. E. Kerr. 2015b. Cow-to-cow variation in fibroblast response to a toll-like receptor 2/6 agonist and its relation to mastitis caused by intramammary challenge with Staphylococcus aureus. Journal of dairy science.

Bhargavan, B., S. M. Woollard, and G. D. Kanmogne. 2016. Toll-like receptor-3 mediates HIV-1 transactivation via NFkappaB and JNK pathways and histone acetylation, but prolonged activation suppresses Tat and HIV-1 replication. Cell Signal 28(2):7-22.

Bhattacharyya, S., U. Gutti, J. Mercado, C. Moore, H. B. Pollard, and R. Biswas. 2011. MAPK signaling pathways regulate IL-8 mRNA stability and IL-8 protein expression in cystic fibrosis lung epithelial cell lines. Am J Physiol Lung Cell Mol Physiol 300(1):L81-87.

Biswas, S. K. and E. Lopez-Collazo. 2009. Endotoxin tolerance: new mechanisms, molecules and clinical significance. Trends in immunology 30(10):475-487.

Blattler, A., L. Yao, H. Witt, Y. Guo, C. M. Nicolet, B. P. Berman, and P. J. Farnham. 2014. Global loss of DNA methylation uncovers intronic enhancers in genes showing expression changes. Genome Biol 15(9):469.

Blum, S., N. Sela, E. D. Heller, S. Sela, and G. Leitner. 2012. Genome analysis of bovine-mastitis-associated Escherichia coli O32:H37 strain P4. J Bacteriol 194(14):3732.

Blum, S. E., E. D. Heller, S. Jacoby, O. Krifucks, and G. Leitner. 2017. Comparison of the immune responses associated with experimental bovine mastitis caused by different strains of Escherichia coli. J Dairy Res 84(2):190-197.

Blum, S. E., E. D. Heller, S. Sela, D. Elad, N. Edery, and G. Leitner. 2015. Genomic and Phenomic Study of Mammary Pathogenic Escherichia coli. PLoS One 10(9):e0136387.

Bougarn, S., P. Cunha, F. B. Gilbert, A. Harmache, G. Foucras, and P. Rainard. 2011. Staphylococcal-associated molecular patterns enhance expression of immune defense genes induced by IL-17 in mammary epithelial cells. Cytokine 56(3):749-759.

Boujenane, I., J. El Aimani, and K. By. 2015. Effects of clinical mastitis on reproductive and milk performance of Holstein cows in Morocco. Trop Anim Health Prod 47(1):207-211.

Bramley, A. J. 1976. Variations in the susceptibility of lactating and non-lactating bovine udders to infection when infused with Escherichia coli. J Dairy Res 43(2):205-211.

Bramley, A. J. and F. H. Dodd. 1984. Reviews of the progress of dairy science: mastitis control--progress and prospects. J Dairy Res 51(3):481-512.

Brand, B., A. Hartmann, D. Repsilber, B. Griesbeck-Zilch, O. Wellnitz, C. Kuhn, S. Ponsuksili, H. H. Meyer, and M. Schwerin. 2011. Comparative expression profiling of E.

coli and S. aureus inoculated primary mammary gland cells sampled from cows with different genetic predispositions for somatic cell score. Genet Sel Evol 43:24.

Brenaut, P., L. Lefevre, A. Rau, D. Laloe, G. Pisoni, P. Moroni, C. Bevilacqua, and P. Martin. 2014. Contribution of mammary epithelial cells to the immune response during early stages of a bacterial infection to Staphylococcus aureus. Vet Res 45:16.

Buckley, C. D., D. Pilling, J. M. Lord, A. N. Akbar, D. Scheel-Toellner, and M. Salmon. 2001. Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation. Trends in immunology 22(4):199-204.

Burvenich, C., D. D. Bannerman, J. D. Lippolis, L. Peelman, B. J. Nonnecke, M. E. Kehrli, Jr., and M. J. Paape. 2007. Cumulative physiological events influence the inflammatory response of the bovine udder to Escherichia coli infections during the transition period. Journal of dairy science 90 Suppl 1:E39-54.

Burvenich, C., V. Van Merris, J. Mehrzad, A. Diez-Fraile, and L. Duchateau. 2003. Severity of E. coli mastitis is mainly determined by cow factors. Vet Res 34(5):521-564.

Cai, D., M. Yuan, H. Liu, Z. Han, S. Pan, Y. Yang, and R. Zhao. 2016. Epigenetic and SP1-mediated regulation is involved in the repression of galactokinase 1 gene in the liver of neonatal piglets born to betaine-supplemented sows. Eur J Nutr.

Callaway, J. B., S. A. Smith, K. P. McKinnon, A. M. de Silva, J. E. Crowe, Jr., and J. P. Ting. 2015. Spleen Tyrosine Kinase (Syk) Mediates IL-1beta Induction by Primary Human Monocytes during Antibody-enhanced Dengue Virus Infection. J Biol Chem 290(28):17306-17320.

Carroll, J. A., N. C. Burdick Sanchez, J. D. Arthingon, C. D. Nelson, A. L. Benjamin, F. T. Korkmaz, D. E. Kerr, and P. A. Lancaster. 2017. In utero exposure to LPS alters the postnatal acute-phase response in beef heifers. Innate Immun 23(1):97-108.

Cavaillon, J. M. 2012. Good and bad fever. Crit Care 16(2):119.

Cha, E., D. Bar, J. A. Hertl, L. W. Tauer, G. Bennett, R. N. Gonzalez, Y. H. Schukken, F. L. Welcome, and Y. T. Grohn. 2011. The cost and management of different types of clinical mastitis in dairy cows estimated by dynamic programming. Journal of dairy science 94(9):4476-4487.

Chang, G., W. Petzl, J. Vanselow, J. Gunther, X. Shen, and H. M. Seyfert. 2015a. Epigenetic mechanisms contribute to enhanced expression of immune response genes in the liver of cows after experimentally induced Escherichia coli mastitis. Vet J 203(3):339-341.

Chang, G., K. Zhang, T. Xu, D. Jin, J. Guo, S. Zhuang, and X. Shen. 2015b. Epigenetic mechanisms contribute to the expression of immune related genes in the livers of dairy cows fed a high concentrate diet. PLoS One 10(4):e0123942.

Chang, G., S. Zhuang, H. M. Seyfert, K. Zhang, T. Xu, D. Jin, J. Guo, and X. Shen. 2015c. Hepatic TLR4 signaling is activated by LPS from digestive tract during SARA, and epigenetic mechanisms contribute to enforced TLR4 expression. Oncotarget 6(36):38578-38590.

Chen, Q., G. He, W. Zhang, T. Xu, H. Qi, J. Li, Y. Zhang, and M. Q. Gao. 2016. Stromal fibroblasts derived from mammary gland of bovine with mastitis display inflammation-specific changes. Sci Rep 6:27462.

Chen, Y. C., C. C. Hsiao, C. J. Chen, T. Y. Chao, S. Y. Leung, S. F. Liu, C. C. Wang, T. Y. Wang, J. C. Chang, C. C. Wu, A. S. Lin, Y. H. Wang, and M. C. Lin. 2014. Aberrant Toll-like receptor 2 promoter methylation in blood cells from patients with pulmonary tuberculosis. J Infect 69(6):546-557.

Cheung, L. W., K. W. Walkiewicz, T. M. Besong, H. Guo, D. H. Hawke, S. T. Arold, and G. B. Mills. 2015. Regulation of the PI3K pathway through a p85alpha monomer-homodimer equilibrium. Elife 4:e06866.

Chow, J. C., D. W. Young, D. T. Golenbock, W. J. Christ, and F. Gusovsky. 1999. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. J Biol Chem 274(16):10689-10692.

Cook, D. N., H. S. Kang, and A. M. Jetten. 2015. Retinoic Acid-Related Orphan Receptors (RORs): Regulatory Functions in Immunity, Development, Circadian Rhythm, and Metabolism. Nucl Receptor Res 2.

Cubeddu, T., C. Cacciotto, S. Pisanu, V. Tedde, A. Alberti, M. Pittau, S. Dore, A. Cannas, S. Uzzau, S. Rocca, and M. F. Addis. 2017. Cathelicidin production and release by mammary epithelial cells during infectious mastitis. Vet Immunol Immunopathol 189:66-70.

De Nardo, D. 2015. Toll-like receptors: Activation, signalling and transcriptional modulation. Cytokine 74(2):181-189.

Deaton, A. M. and A. Bird. 2011. CpG islands and the regulation of transcription. Genes & development 25(10):1010-1022.

Depino, A. M. 2015. Early prenatal exposure to LPS results in anxiety- and depression-related behaviors in adulthood. Neuroscience 299:56-65.

Diez-Fraile, A., E. Meyer, and C. Burvenich. 2002. Regulation of adhesion molecules on circulating neutrophils during coliform mastitis and their possible immunomodulation with drugs. Vet Immunol Immunopathol 86(1-2):1-10.

Doherty, R., C. O'Farrelly, and K. G. Meade. 2013. Epigenetic regulation of the innate immune response to LPS in bovine peripheral blood mononuclear cells (PBMC). Vet Immunol Immunopathol 154(3-4):102-110.

Doherty, R., R. Whiston, P. Cormican, E. K. Finlay, C. Couldrey, C. Brady, C. O'Farrelly, and K. G. Meade. 2016. The CD4(+) T cell methylome contributes to a distinct CD4(+) T cell transcriptional signature in Mycobacterium bovis-infected cattle. Sci Rep 6:31014.

Dosogne, H., C. Burvenich, T. van Werven, E. Roets, E. N. Noordhuizen-Stassen, and B. Goddeeris. 1997. Increased surface expression of CD11b receptors on polymorphonuclear leukocytes is not sufficient to sustain phagocytosis during Escherichia coli mastitis in early postpartum dairy cows. Vet Immunol Immunopathol 60(1-2):47-59.

Drury, R. E., D. O'Connor, and A. J. Pollard. 2017. The Clinical Application of MicroRNAs in Infectious Disease. Front Immunol 8:1182.

Duran Aguilar, M., S. I. Roman Ponce, F. J. Ruiz Lopez, E. Gonzalez Padilla, C. G. Vasquez Pelaez, A. Bagnato, and M. G. Strillacci. 2017. Genome-wide association study for milk somatic cell score in holstein cattle using copy number variation as markers. J Anim Breed Genet 134(1):49-59.

El Gazzar, M., B. K. Yoza, J. Y. Hu, S. L. Cousart, and C. E. McCall. 2007. Epigenetic silencing of tumor necrosis factor alpha during endotoxin tolerance. The Journal of biological chemistry 282(37):26857-26864.

Elazar, S., E. Gonen, A. Livneh-Kol, I. Rosenshine, and N. Y. Shpigel. 2010. Neutrophil recruitment in endotoxin-induced murine mastitis is strictly dependent on mammary alveolar macrophages. Vet Res 41(1):10.

Ellis, S., A. Mouihate, and Q. J. Pittman. 2005. Early life immune challenge alters innate immune responses to lipopolysaccharide: implications for host defense as adults. FASEB J 19(11):1519-1521.

Elsasser, T. H., J. W. Blum, and S. Kahl. 2005. Characterization of calves exhibiting a novel inheritable TNF-alpha hyperresponsiveness to endotoxin: associations with increased pathophysiological complications. J Appl Physiol (1985) 98(6):2045-2055.

Ettou, S., C. Humbrecht, B. Benet, K. Billot, D. d'Allard, V. Mariot, M. Goodhardt, O. Kosmider, P. Mayeux, E. Solary, and M. Fontenay. 2013. Epigenetic control of NF-kappaB-dependent FAS gene transcription during progression of myelodysplastic syndromes. Mol Cancer Res 11(7):724-735.

Fan, H. and J. A. Cook. 2004. Molecular mechanisms of endotoxin tolerance. Journal of endotoxin research 10(2):71-84.

Franceschi, C., M. Bonafe, S. Valensin, F. Olivieri, M. De Luca, E. Ottaviani, and G. De Benedictis. 2000. Inflamm-aging. An evolutionary perspective on immunosenescence. Ann N Y Acad Sci 908:244-254.

Fuenzalida, M. J., P. M. Fricke, and P. L. Ruegg. 2015. The association between occurrence and severity of subclinical and clinical mastitis on pregnancies per artificial insemination at first service of Holstein cows. Journal of dairy science 98(6):3791-3805.

Ganda, E. K., R. S. Bisinotto, D. H. Decter, and R. C. Bicalho. 2016. Evaluation of an On-Farm Culture System (Accumast) for Fast Identification of Milk Pathogens Associated with Clinical Mastitis in Dairy Cows. PLoS One 11(5):e0155314.

Gilbert, F. B., P. Cunha, K. Jensen, E. J. Glass, G. Foucras, C. Robert-Granie, R. Rupp, and P. Rainard. 2013. Differential response of bovine mammary epithelial cells to Staphylococcus aureus or Escherichia coli agonists of the innate immune system. Vet. Res. 44:40.

Giri, S. N., P. Emau, J. S. Cullor, G. H. Stabenfeldt, M. L. Bruss, R. H. Bondurant, and B. I. Osburn. 1990. Effects of endotoxin infusion on circulating levels of eicosanoids, progesterone, cortisol, glucose and lactic acid, and abortion in pregnant cows. Veterinary microbiology 21(3):211-231.

Glynn, D. J., M. R. Hutchinson, and W. V. Ingman. 2014. Toll-like receptor 4 regulates lipopolysaccharide-induced inflammation and lactation insufficiency in a mouse model of mastitis. Biol Reprod 90(5):91.

Gomes, A. V., S. M. de Souza Morais, S. L. Menezes-Filho, L. G. de Almeida, R. P. Rocha, J. M. Siqueira Ferreira, L. L. Dos Santos, L. C. Malaquias, and L. F. Coelho. 2016. The demethylation profile of the TNF-alpha promoter gene is associated with high expression of this cytokine in Dengue virus patients. J Med Virol.

Gopisetty, G., J. Xu, D. Sampath, H. Colman, and V. K. Puduvalli. 2013. Epigenetic regulation of CD133/PROM1 expression in glioma stem cells by Sp1/myc and promoter methylation. Oncogene 32(26):3119-3129.

Gou, Z., R. Liu, G. Zhao, M. Zheng, P. Li, H. Wang, Y. Zhu, J. Chen, and J. Wen. 2012. Epigenetic modification of TLRs in leukocytes is associated with increased susceptibility to Salmonella enteritidis in chickens. PLoS One 7(3):e33627.

Govignon-Gion, A., R. Dassonneville, G. Baloche, and V. Ducrocq. 2015. Multiple trait genetic evaluation of clinical mastitis in three dairy cattle breeds. Animal:1-8.

Govignon-Gion, A., R. Dassonneville, G. Baloche, and V. Ducrocq. 2016. Multiple trait genetic evaluation of clinical mastitis in three dairy cattle breeds. Animal 10(4):558-565.

Gowers, I. R., K. Walters, E. Kiss-Toth, R. C. Read, G. W. Duff, and A. G. Wilson. 2011. Age-related loss of CpG methylation in the tumour necrosis factor promoter. Cytokine 56(3):792-797.

Green, B. B., S. Kandasamy, T. H. Elsasser, and D. E. Kerr. 2011a. The use of dermal fibroblasts as a predictive tool of the toll-like receptor 4 response pathway and its development in Holstein heifers. Journal of dairy science 94(11):5502-5514.

Green, B. B., S. Kandasamy, T. H. Elsasser, and D. E. Kerr. 2011b. The use of dermal fibroblasts as a predictive tool of the toll-like receptor 4 response pathway and its development in Holstein heifers. J. Dairy Sci. 94(11):5502-5514.

Green, B. B. and D. E. Kerr. 2014. Epigenetic contribution to individual variation in response to lipopolysaccharide in bovine dermal fibroblasts. Vet Immunol Immunopathol 157(1-2):49-58.

Green, B. B., S. D. McKay, and D. E. Kerr. 2015. Age dependent changes in the LPS induced transcriptome of bovine dermal fibroblasts occurs without major changes in the methylome. BMC Genomics 16:30.

Grohn, Y. T., D. J. Wilson, R. N. Gonzalez, J. A. Hertl, H. Schulte, G. Bennett, and Y. H. Schukken. 2004. Effect of pathogen-specific clinical mastitis on milk yield in dairy cows. J. Dairy Sci. 87(10):3358-3374.

Gunther, J., A. Czabanska, I. Bauer, J. A. Leigh, O. Holst, and H. M. Seyfert. 2016a. Streptococcus uberis strains isolated from the bovine mammary gland evade immune recognition by mammary epithelial cells, but not of macrophages. Vet Res 47:13.

Gunther, J., M. Koy, A. Berthold, H. J. Schuberth, and H. M. Seyfert. 2016b. Comparison of the pathogen species-specific immune response in udder derived cell types and their models. Vet Res 47:22.

Gunther, J., W. Petzl, I. Bauer, S. Ponsuksili, H. Zerbe, H. J. Schuberth, R. M. Brunner, and H. M. Seyfert. 2017a. Differentiating Staphylococcus aureus from Escherichia coli mastitis: S. aureus triggers unbalanced immune-dampening and host cell invasion immediately after udder infection. Sci Rep 7(1):4811.

Gunther, J., W. Petzl, H. Zerbe, H. J. Schuberth, D. Koczan, L. Goetze, and H. M. Seyfert. 2012. Lipopolysaccharide priming enhances expression of effectors of immune defence while decreasing expression of pro-inflammatory cytokines in mammary epithelia cells from cows. BMC Genomics 13:17.

Gunther, J., W. Petzl, H. Zerbe, H. J. Schuberth, and H. M. Seyfert. 2017b. TLR ligands, but not modulators of histone modifiers, can induce the complex immune response pattern of endotoxin tolerance in mammary epithelial cells. Innate Immun 23(2):155-164.

Halim, T. Y., A. MacLaren, M. T. Romanish, M. J. Gold, K. M. McNagny, and F. Takei. 2012. Retinoic-acid-receptor-related orphan nuclear receptor alpha is required for natural helper cell development and allergic inflammation. Immunity 37(3):463-474.

Hawkins, P. T. and L. R. Stephens. 2015. PI3K signalling in inflammation. Biochim Biophys Acta 1851(6):882-897.

Hellwig, S., C. V. Miduturu, S. Kanda, J. Zhang, P. Filippakopoulos, E. Salah, X. Deng, H. G. Choi, W. Zhou, W. Hur, S. Knapp, N. S. Gray, and T. E. Smithgall. 2012. Small-molecule inhibitors of the c-Fes protein-tyrosine kinase. Chem Biol 19(4):529-540.

Hennessy, C. and D. P. McKernan. 2016. Epigenetics and innate immunity: the 'unTolld' story. Immunol Cell Biol 94(7):631-639.

Herry, V., C. Gitton, G. Tabouret, M. Reperant, L. Forge, C. Tasca, F. B. Gilbert, E. Guitton, C. Barc, C. Staub, D. G. E. Smith, P. Germon, G. Foucras, and P. Rainard. 2017. Local immunization impacts the response of dairy cows to Escherichia coli mastitis. Sci Rep 7(1):3441.

Hertl, J. A., Y. H. Schukken, F. L. Welcome, L. W. Tauer, and Y. T. Grohn. 2014. Effects of pathogen-specific clinical mastitis on probability of conception in Holstein dairy cows. Journal of dairy science 97(11):6942-6954.

Hillerton, J. E., A. J. Bramley, R. T. Staker, and C. H. McKinnon. 1995. Patterns of intramammary infection and clinical mastitis over a 5 year period in a closely monitored herd applying mastitis control measures. J Dairy Res 62(1):39-50.

Hillreiner, M., C. Schmautz, I. Ballweg, V. Korenkova, M. W. Pfaffl, and H. Kliem. 2017. Gene expression profiling in pbMEC - in search of molecular biomarkers to predict immunoglobulin production in bovine milk. BMC Vet Res 13(1):369.

Hodyl, N. A., K. M. Krivanek, E. Lawrence, V. L. Clifton, and D. M. Hodgson. 2007. Prenatal exposure to a pro-inflammatory stimulus causes delays in the development of the innate immune response to LPS in the offspring. J. Neuroimmunol. 190(1-2):61-71.

Hollox, E. J. and B. P. Hoh. 2014. Human gene copy number variation and infectious disease. Hum Genet 133(10):1217-1233.

Holsapple, M. P., L. J. West, and K. S. Landreth. 2003. Species comparison of anatomical and functional immune system development. Birth defects research. Part B, Developmental and reproductive toxicology 68(4):321-334.

Huang da, W., B. T. Sherman, and R. A. Lempicki. 2009a. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 37(1):1-13.

Huang da, W., B. T. Sherman, and R. A. Lempicki. 2009b. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature protocols 4(1):44-57.

Ibeagha-Awemu, E. M., J. W. Lee, A. E. Ibeagha, D. D. Bannerman, M. J. Paape, and X. Zhao. 2008. Bacterial lipopolysaccharide induces increased expression of toll-like receptor (TLR) 4 and downstream TLR signaling molecules in bovine mammary epithelial cells. Vet. Res. 39(2):11.

Ichiyama, K., T. Chen, X. Wang, X. Yan, B. S. Kim, S. Tanaka, D. Ndiaye-Lobry, Y. Deng, Y. Zou, P. Zheng, Q. Tian, I. Aifantis, L. Wei, and C. Dong. 2015. The methylcytosine dioxygenase Tet2 promotes DNA demethylation and activation of cytokine gene expression in T cells. Immunity 42(4):613-626.

Jahan, N., A. Minuti, and E. Trevisi. 2015. Assessment of immune response in periparturient dairy cows using ex vivo whole blood stimulation assay with

lipopolysaccharides and carrageenan skin test. Veterinary immunology and immunopathology 165(3-4):119-126.

Jamrozik, J. and L. R. Schaeffer. 2012. Test-day somatic cell score, fat-to-protein ratio and milk yield as indicator traits for sub-clinical mastitis in dairy cattle. J Anim Breed Genet 129(1):11-19.

Jian, Z. J., Z. Yang, M. S. Miller, C. D. Carter, D. O. Slauson, and P. N. Bochsler. 1995. Interleukin-6 secretion by bacterial lipopolysaccharide-stimulated bovine alveolar macrophages in vitro. Veterinary immunology and immunopathology 49(1-2):51-60.

Jones, K. J., S. Ekhlassi, D. Montufar-Solis, J. R. Klein, and J. S. Schaefer. 2010. Differential cytokine patterns in mouse macrophages and gingival fibroblasts after stimulation with porphyromonas gingivalis or Escherichia coli lipopolysaccharide. Journal of periodontology 81(12):1850-1857.

Kadri, N. K., B. Guldbrandtsen, M. S. Lund, and G. Sahana. 2015. Genetic dissection of milk yield traits and mastitis resistance quantitative trait loci on chromosome 20 in dairy cattle. Journal of dairy science 98(12):9015-9025.

Kalinski, P. 2012. Regulation of immune responses by prostaglandin E2. J Immunol 188(1):21-28.

Kandasamy, S., B. B. Green, A. L. Benjamin, and D. E. Kerr. 2011. Between-cow variation in dermal fibroblast response to lipopolysaccharide reflected in resolution of inflammation during Escherichia coli mastitis. Journal of dairy science 94(12):5963-5975.

Kandasamy, S. and D. E. Kerr. 2012. Genomic analysis of between-cow variation in dermal fibroblast response to lipopolysaccharide. Journal of dairy science 95(7):3852-3864.

Kang, H. S., K. Okamoto, Y. Takeda, J. Y. Beak, K. Gerrish, C. D. Bortner, L. M. DeGraff, T. Wada, W. Xie, and A. M. Jetten. 2011. Transcriptional profiling reveals a role for RORalpha in regulating gene expression in obesity-associated inflammation and hepatic steatosis. Physiol Genomics 43(13):818-828.

Kanzleiter, T., M. Jahnert, G. Schulze, J. Selbig, N. Hallahan, R. W. Schwenk, and A. Schurmann. 2015. Exercise training alters DNA methylation patterns in genes related to muscle growth and differentiation in mice. American journal of physiology. Endocrinology and metabolism 308(10):E912-920.

Kawai, T. and S. Akira. 2007. Signaling to NF-kappaB by Toll-like receptors. Trends Mol. Med. 13(11):460-469.

Keestra-Gounder, A. M., M. X. Byndloss, N. Seyffert, B. M. Young, A. Chavez-Arroyo, A. Y. Tsai, S. A. Cevallos, M. G. Winter, O. H. Pham, C. R. Tiffany, M. F. de Jong, T. Kerrinnes, R. Ravindran, P. A. Luciw, S. J. McSorley, A. J. Baumler, and R. M. Tsolis. 2016. NOD1 and NOD2 signalling links ER stress with inflammation. Nature 532(7599):394-397.

Kirsten, T. B., B. P. de Oliveira, A. P. de Oliveira, K. Kieling, W. T. de Lima, J. Palermo-Neto, and M. M. Bernardi. 2011. Single early prenatal lipopolysaccharide exposure prevents subsequent airway inflammation response in an experimental model of asthma. Life Sci 89(1-2):15-19.

Koga, Y., T. Hisada, T. Ishizuka, M. Utsugi, A. Ono, M. Yatomi, Y. Kamide, H. Aoki-Saito, H. Tsurumaki, K. Dobashi, and M. Yamada. 2016. CREB regulates TNF-alpha-induced GM-CSF secretion via p38 MAPK in human lung fibroblasts. Allergol Int 65(4):406-413.

Kojima, A., T. Kobayashi, S. Ito, A. Murasawa, K. Nakazono, and H. Yoshie. 2015. Tumor necrosis factor-alpha gene promoter methylation in Japanese adults with chronic periodontitis and rheumatoid arthritis. Journal of periodontal research.

Kok, B. P. and E. Saez. 2014. Activating PI3-kinase to dampen inflammation. Chem Biol 21(8):917-918.

Kornalijnslijper, E., B. Beerda, I. Daemen, J. van der Werf, T. van Werven, T. Niewold, V. Rutten, and E. Noordhuizen-Stassen. 2003. The effect of milk production level on host resistance of dairy cows, as assessed by the severity of experimental Escherichia coli mastitis. Vet Res 34(6):721-736.

Kox, M., S. de Kleijn, J. C. Pompe, B. P. Ramakers, M. G. Netea, J. G. van der Hoeven, C. W. Hoedemaekers, and P. Pickkers. 2011. Differential ex vivo and in vivo endotoxin tolerance kinetics following human endotoxemia. Crit. Care Med. 39(8):1866-1870.

Kumar, H., T. Kawai, and S. Akira. 2011. Pathogen recognition by the innate immune system. Int Rev Immunol 30(1):16-34.

Labzin, L. I., S. V. Schmidt, S. L. Masters, M. Beyer, W. Krebs, K. Klee, R. Stahl, D. Lutjohann, J. L. Schultze, E. Latz, and D. De Nardo. 2015. ATF3 Is a Key Regulator of Macrophage IFN Responses. J Immunol 195(9):4446-4455.

Lacerte, P., A. Brunet, B. Egarnes, B. Duchene, J. P. Brown, and J. Gosselin. 2016. Overexpression of TLR2 and TLR9 on monocyte subsets of active rheumatoid arthritis patients contributes to enhance responsiveness to TLR agonists. Arthritis Res Ther 18(1):10.

Lago, A., S. M. Godden, R. Bey, P. L. Ruegg, and K. Leslie. 2011a. The selective treatment of clinical mastitis based on on-farm culture results: I. Effects on antibiotic use, milk withholding time, and short-term clinical and bacteriological outcomes. Journal of dairy science 94(9):4441-4456.

Lago, A., S. M. Godden, R. Bey, P. L. Ruegg, and K. Leslie. 2011b. The selective treatment of clinical mastitis based on on-farm culture results: II. Effects on lactation performance, including clinical mastitis recurrence, somatic cell count, milk production, and cow survival. Journal of dairy science 94(9):4457-4467.

Lasala, N. and H. Zhou. 2007. Effects of maternal exposure to LPS on the inflammatory response in the offspring. Journal of neuroimmunology 189(1-2):95-101.

Lee, G. L., J. Y. Wu, C. C. Yeh, and C. C. Kuo. 2016. TLR4 induces CREB-mediated IL-6 production via upregulation of F-spondin to promote vascular smooth muscle cell migration. Biochem Biophys Res Commun 473(4):1205-1210.

Lee, J. W., D. D. Bannerman, M. J. Paape, M. K. Huang, and X. Zhao. 2006. Characterization of cytokine expression in milk somatic cells during intramammary infections with Escherichia coli or Staphylococcus aureus by real-time PCR. Vet Res 37(2):219-229.

Lee, S. M., E. J. Kim, K. Suk, and W. H. Lee. 2011. BAFF and APRIL induce inflammatory activation of THP-1 cells through interaction with their conventional receptors and activation of MAPK and NF-kappaB. Inflamm Res 60(9):807-815.

Leimbach, A., A. Poehlein, J. Vollmers, D. Gorlich, R. Daniel, and U. Dobrindt. 2017. No evidence for a bovine mastitis Escherichia coli pathotype. BMC Genomics 18(1):359.

Lemaitre, B., E. Nicolas, L. Michaut, J. M. Reichhart, and J. A. Hoffmann. 1996. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell 86(6):973-983.

Lents, C. A., R. P. Wettemann, M. J. Paape, J. A. Vizcarra, M. L. Looper, D. S. Buchanan, and K. S. Lusby. 2002. Efficacy of intramuscular treatment of beef cows with oxytetracycline to reduce mastitis and to increase calf growth. Journal of animal science 80(6):1405-1412.

Li, J., R. Li, Y. Wang, X. Hu, Y. Zhao, L. Li, C. Feng, X. Gu, F. Liang, S. J. Lamont, S. Hu, H. Zhou, and N. Li. 2015. Genome-wide DNA methylome variation in two genetically distinct chicken lines using MethylC-seq. BMC Genomics 16:851.

Liang, D., L. M. Arnold, C. J. Stowe, R. J. Harmon, and J. M. Bewley. 2017. Estimating US dairy clinical disease costs with a stochastic simulation model. Journal of dairy science 100(2):1472-1486.

Lu, Y. C., W. C. Yeh, and P. S. Ohashi. 2008. LPS/TLR4 signal transduction pathway. Cytokine 42(2):145-151.

Lund, M. S., J. Jensen, and P. H. Petersen. 1999. Estimation of genetic and phenotypic parameters for clinical mastitis, somatic cell production deviance, and protein yield in dairy cattle using Gibbs sampling. Journal of dairy science 82(5):1045-1051.

Magee, D. A., M. Taraktsoglou, K. E. Killick, N. C. Nalpas, J. A. Browne, S. D. Park, K. M. Conlon, D. J. Lynn, K. Hokamp, S. V. Gordon, E. Gormley, and D. E. MacHugh. 2012. Global gene expression and systems biology analysis of bovine monocyte-derived macrophages in response to in vitro challenge with Mycobacterium bovis. PLoS ONE 7(2):e32034.

Magnusson, M., R. Tobes, J. Sancho, and E. Pareja. 2007. Cutting edge: natural DNA repetitive extragenic sequences from gram-negative pathogens strongly stimulate TLR9. J Immunol 179(1):31-35.

Makovec, J. A. and P. L. Ruegg. 2003. Results of milk samples submitted for microbiological examination in Wisconsin from 1994 to 2001. Journal of dairy science 86(11):3466-3472.

Mann, M., A. Mehta, J. L. Zhao, K. Lee, G. K. Marinov, Y. Garcia-Flores, and D. Baltimore. 2017. An NF-kappaB-microRNA regulatory network tunes macrophage inflammatory responses. Nat Commun 8(1):851.

Marttila, S., L. Kananen, S. Hayrynen, J. Jylhava, T. Nevalainen, A. Hervonen, M. Jylha, M. Nykter, and M. Hurme. 2015. Ageing-associated changes in the human DNA methylome: genomic locations and effects on gene expression. BMC Genomics 16:179.

Meade, K. G., G. M. O'Gorman, F. Narciandi, D. E. Machugh, and C. O'Farrelly. 2012. Functional characterisation of bovine interleukin 8 promoter haplotypes in vitro. Mol Immunol 50(1-2):108-116.

Mehrzad, J., L. Duchateau, and C. Burvenich. 2004. Viability of milk neutrophils and severity of bovine coliform mastitis. Journal of dairy science 87(12):4150-4162.

Mehrzad, J., L. Duchateau, S. Pyorala, and C. Burvenich. 2002. Blood and milk neutrophil chemiluminescence and viability in primiparous and pluriparous dairy cows during late pregnancy, around parturition and early lactation. Journal of dairy science 85(12):3268-3276.

Mehrzad, J., D. Janssen, L. Duchateau, and C. Burvenich. 2008. Increase in Escherichia coli inoculum dose accelerates CD8+ T-cell trafficking in the primiparous bovine mammary gland. Journal of dairy science 91(1):193-201.

Michel, T., J. M. Reichhart, J. A. Hoffmann, and J. Royet. 2001. Drosophila Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. Nature 414(6865):756-759.

Miller, R. B., S. D. Van Camp, and D. A. Barnum. 1983. The effects of intra-amniotic inoculation of Hemophilus somnus on the bovine fetus and dam. Veterinary pathology 20(5):574-583.

Mills, J. A., D. S. Zarlenga, P. L. Habecker, and R. M. Dyer. 2009. Age, segment, and horn disease affect expression of cytokines, growth factors, and receptors in the epidermis and dermis of the bovine claw. J. Dairy Sci. 92(12):5977-5987.

Mohammed-Ali, Z., G. L. Cruz, and J. G. Dickhout. 2015. Crosstalk between the unfolded protein response and NF-kappaB-mediated inflammation in the progression of chronic kidney disease. J Immunol Res 2015:428508.

Moore, L. D., T. Le, and G. Fan. 2013. DNA methylation and its basic function. Neuropsychopharmacology 38(1):23-38.

Moretto, N., S. Bertolini, C. Iadicicco, G. Marchini, M. Kaur, G. Volpi, R. Patacchini, D. Singh, and F. Facchinetti. 2012. Cigarette smoke and its component acrolein augment IL-8/CXCL8 mRNA stability via p38 MAPK/MK2 signaling in human pulmonary cells. Am J Physiol Lung Cell Mol Physiol 303(10):L929-938.

Mosser, D. M. and X. Zhang. 2008. Interleukin-10: new perspectives on an old cytokine. Immunol Rev 226:205-218.

Munoz, L. E., M. Leppkes, T. A. Fuchs, M. Hoffmann, and M. Herrmann. 2017. Missing in action-The meaning of cell death in tissue damage and inflammation. Immunol Rev 280(1):26-40.

Nahid, M. A., K. M. Pauley, M. Satoh, and E. K. Chan. 2009. miR-146a is critical for endotoxin-induced tolerance: IMPLICATION IN INNATE IMMUNITY. J Biol Chem 284(50):34590-34599.

Nakada, T. A., J. A. Russell, J. H. Boyd, and K. R. Walley. 2011. IL17A genetic variation is associated with altered susceptibility to Gram-positive infection and mortality of severe sepsis. Crit Care 15(5):R254.

Netea, M. G., L. A. Joosten, E. Latz, K. H. Mills, G. Natoli, H. G. Stunnenberg, L. A. O'Neill, and R. J. Xavier. 2016. Trained immunity: A program of innate immune memory in health and disease. Science 352(6284):aaf1098.

Nile, C. J., R. C. Read, M. Akil, G. W. Duff, and A. G. Wilson. 2008. Methylation status of a single CpG site in the IL6 promoter is related to IL6 messenger RNA levels and rheumatoid arthritis. Arthritis Rheum 58(9):2686-2693.

Novak, K. 2014. Functional polymorphisms in Toll-like receptor genes for innate immunity in farm animals. Veterinary immunology and immunopathology 157(1-2):1-11.

Nyati, K. K., K. Masuda, M. M. Zaman, P. K. Dubey, D. Millrine, J. P. Chalise, M. Higa, S. Li, D. M. Standley, K. Saito, H. Hanieh, and T. Kishimoto. 2017. TLR4-induced NF-kappaB and MAPK signaling regulate the IL-6 mRNA stabilizing protein Arid5a. Nucleic Acids Res 45(5):2687-2703.

Oliveira, C. S., H. Hogeveen, A. M. Botelho, P. V. Maia, S. G. Coelho, and J. P. Haddad. 2015. Cow-specific risk factors for clinical mastitis in Brazilian dairy cattle. Prev Vet Med 121(3-4):297-305.

Oliveira, L., C. Hulland, and P. L. Ruegg. 2013. Characterization of clinical mastitis occurring in cows on 50 large dairy herds in Wisconsin. Journal of dairy science 96(12):7538-7549.

Oliver, S. P., B. E. Gillespie, S. J. Headrick, H. Moorehead, P. Lunn, H. H. Dowlen, D. L. Johnson, K. C. Lamar, S. T. Chester, and W. M. Moseley. 2004. Efficacy of extended ceftiofur intramammary therapy for treatment of subclinical mastitis in lactating dairy cows. Journal of dairy science 87(8):2393-2400.

Paibomesai, M., B. Hussey, M. Nino-Soto, and B. A. Mallard. 2013. Effects of parturition and dexamethasone on DNA methylation patterns of IFN-gamma and IL-4 promoters in CD4+ T-lymphocytes of Holstein dairy cows. Can J Vet Res 77(1):54-62.

Pan, H., L. Chen, S. Dogra, A. L. Teh, J. H. Tan, Y. I. Lim, Y. C. Lim, S. Jin, Y. K. Lee, P. Y. Ng, M. L. Ong, S. Barton, Y. S. Chong, M. J. Meaney, P. D. Gluckman, W. Stunkel, C. Ding, and J. D. Holbrook. 2012. Measuring the methylome in clinical samples: improved processing of the Infinium Human Methylation450 BeadChip Array. Epigenetics 7(10):1173-1187.

Pang, M., L. Sun, T. He, H. Bao, L. Zhang, Y. Zhou, H. Zhang, R. Wei, Y. Liu, and R. Wang. 2017. Molecular and virulence characterization of highly prevalent Streptococcus agalactiae circulated in bovine dairy herds. Vet Res 48(1):65.

Pareek, R., O. Wellnitz, R. Van Dorp, J. Burton, and D. Kerr. 2005. Immunorelevant gene expression in LPS-challenged bovine mammary epithelial cells. J Appl Genet 46(2):171-177.

Parihar, A., T. D. Eubank, and A. I. Doseff. 2010. Monocytes and macrophages regulate immunity through dynamic networks of survival and cell death. Journal of innate immunity 2(3):204-215.

Payne, B., Bradley, J.A., Coombes, E., Lusby, E., Mining, K. Hunt, C., Bradley, A.J. 2013. The Aetiology of Bovine Mastitis in UK Dairy Herds. Pages 17-18, 59-60 in Proceeding of the British Mastitis Conference.

Pechous, R. D. 2017. With Friends Like These: The Complex Role of Neutrophils in the Progression of Severe Pneumonia. Front Cell Infect Microbiol 7:160.

Pena, C. J., Y. D. Neugut, and F. A. Champagne. 2013. Developmental timing of the effects of maternal care on gene expression and epigenetic regulation of hormone receptor levels in female rats. Endocrinology 154(11):4340-4351.

Persson Waller, K., Y. Persson, A. K. Nyman, and L. Stengarde. 2014. Udder health in beef cows and its association with calf growth. Acta Vet Scand 56:9.

Petzl, W., J. Gunther, K. Muhlbauer, H. M. Seyfert, H. J. Schuberth, J. Hussen, C. Sauter-Louis, A. Hafner-Marx, and H. Zerbe. 2016. Early transcriptional events in the udder and teat after intra-mammary Escherichia coli and Staphylococcus aureus challenge. Innate Immun 22(4):294-304.

Petzl, W., J. Gunther, T. Pfister, C. Sauter-Louis, L. Goetze, S. von Aulock, A. Hafner-Marx, H. J. Schuberth, H. M. Seyfert, and H. Zerbe. 2012. Lipopolysaccharide

pretreatment of the udder protects against experimental Escherichia coli mastitis. Innate Immun 18(3):467-477.

Petzl, W., H. Zerbe, J. Gunther, W. Yang, H. M. Seyfert, G. Nurnberg, and H. J. Schuberth. 2008. Escherichia coli, but not Staphylococcus aureus triggers an early increased expression of factors contributing to the innate immune defense in the udder of the cow. Vet Res 39(2):18.

Pociask, D. A., E. V. Scheller, S. Mandalapu, K. J. McHugh, R. I. Enelow, C. L. Fattman, J. K. Kolls, and J. F. Alcorn. 2013. IL-22 is essential for lung epithelial repair following influenza infection. Am J Pathol 182(4):1286-1296.

Porcherie, A., F. B. Gilbert, P. Germon, P. Cunha, A. Trotereau, C. Rossignol, N. Winter, P. Berthon, and P. Rainard. 2016. IL-17A Is an Important Effector of the Immune Response of the Mammary Gland to Escherichia coli Infection. J Immunol 196(2):803-812.

Quiros, M., H. Nishio, P. A. Neumann, D. Siuda, J. C. Brazil, V. Azcutia, R. Hilgarth, M. N. O'Leary, V. Garcia-Hernandez, G. Leoni, M. Feng, G. Bernal, H. Williams, P. H. Dedhia, C. Gerner-Smidt, J. Spence, C. A. Parkos, T. L. Denning, and A. Nusrat. 2017. Macrophage-derived IL-10 mediates mucosal repair by epithelial WISP-1 signaling. J Clin Invest 127(9):3510-3520.

Rainard, P., P. Cunha, and F. B. Gilbert. 2016. Innate and Adaptive Immunity Synergize to Trigger Inflammation in the Mammary Gland. PLoS One 11(4):e0154172.

Rainard, P. and C. Riollet. 2006. Innate immunity of the bovine mammary gland. Vet Res 37(3):369-400.

Rehbein, P., K. Brugemann, T. Yin, V. B. UK, X. L. Wu, and S. Konig. 2013. Inferring relationships between clinical mastitis, productivity and fertility: a recursive model application including genetics, farm associated herd management, and cow-specific antibiotic treatments. Prev Vet Med 112(1-2):58-67.

Revelo, X. S. and M. R. Waldron. 2012. In vitro effects of Escherichia coli lipopolysaccharide on the function and gene expression of neutrophils isolated from the blood of dairy cows. Journal of dairy science 95(5):2422-2441.

Rinchai, D., P. Khaenam, C. Kewcharoenwong, S. Buddhisa, R. Pankla, D. Chaussabel, G. J. Bancroft, and G. Lertmemongkolchai. 2012. Production of interleukin-27 by human neutrophils regulates their function during bacterial infection. Eur J Immunol 42(12):3280-3290.

Rogers, K. M., D. H. Black, and R. Eberle. 2007. Primary mouse dermal fibroblast cell cultures as an in vitro model system for the differential pathogenicity of cross-species herpesvirus papio 2 infections. Arch Virol 152(3):543-552.

Rollin, E., K. C. Dhuyvetter, and M. W. Overton. 2015. The cost of clinical mastitis in the first 30 days of lactation: An economic modeling tool. Prev Vet Med 122(3):257-264.

Rossato, M., G. Curtale, N. Tamassia, M. Castellucci, L. Mori, S. Gasperini, B. Mariotti, M. De Luca, M. Mirolo, M. A. Cassatella, M. Locati, and F. Bazzoni. 2012. IL-10-induced microRNA-187 negatively regulates TNF-alpha, IL-6, and IL-12p40 production in TLR4-stimulated monocytes. Proc Natl Acad Sci U S A 109(45):E3101-3110.

Roussel, P., P. Cunha, A. Porcherie, W. Petzl, F. B. Gilbert, C. Riollet, H. Zerbe, P. Rainard, and P. Germon. 2015. Investigating the contribution of IL-17A and IL-17F to the host response during Escherichia coli mastitis. Vet Res 46:56.

Roussel, P., A. Porcherie, M. Reperant-Ferter, P. Cunha, C. Gitton, P. Rainard, and P. Germon. 2017. Escherichia coli mastitis strains: In vitro phenotypes and severity of infection in vivo. PLoS One 12(7):e0178285.

Rudolf, R., R. Busch, A. K. Patra, K. Muhammad, A. Avots, J. C. Andrau, S. Klein-Hessling, and E. Serfling. 2014. Architecture and expression of the nfatc1 gene in lymphocytes. Front Immunol 5:21.

Ruegg, P. L. 2017. A 100-Year Review: Mastitis detection, management, and prevention. Journal of dairy science 100(12):10381-10397.

Runyon, R. S., L. M. Cachola, N. Rajeshuni, T. Hunter, M. Garcia, R. Ahn, F. Lurmann, R. Krasnow, L. M. Jack, R. L. Miller, G. E. Swan, A. Kohli, A. C. Jacobson, and K. C. Nadeau. 2012. Asthma discordance in twins is linked to epigenetic modifications of T cells. PLoS One 7(11):e48796.

Rupp, R. and D. Boichard. 2003. Genetics of resistance to mastitis in dairy cattle. Vet Res 34(5):671-688.

Sacramento, L., S. C. Trevelin, M. S. Nascimento, D. S. Lima-Junior, D. L. Costa, R. P. Almeida, F. Q. Cunha, J. S. Silva, and V. Carregaro. 2015. Toll-like receptor 9 signaling in dendritic cells regulates neutrophil recruitment to inflammatory foci following Leishmania infantum infection. Infect Immun 83(12):4604-4616.

Salazar-Camarena, D. C., P. C. Ortiz-Lazareno, A. Cruz, E. Oregon-Romero, J. R. Machado-Contreras, J. F. Munoz-Valle, M. Orozco-Lopez, M. Marin-Rosales, and C. A. Palafox-Sanchez. 2016. Association of BAFF, APRIL serum levels, BAFF-R, TACI and BCMA expression on peripheral B-cell subsets with clinical manifestations in systemic lupus erythematosus. Lupus 25(6):582-592.

Sandberg, W. J., K. Otterdal, L. Gullestad, B. Halvorsen, A. Ragnarsson, S. S. Froland, J. K. Damas, E. Oie, P. Aukrust, G. K. Hansson, and A. Yndestad. 2009. The tumour necrosis factor superfamily ligand APRIL (TNFSF13) is released upon platelet activation and expressed in atherosclerosis. Thromb Haemost 102(4):704-710.

Schaub, B., J. Liu, S. Hoppler, I. Schleich, J. Huehn, S. Olek, G. Wieczorek, S. Illi, and E. von Mutius. 2009. Maternal farm exposure modulates neonatal immune mechanisms through regulatory T cells. J Allergy Clin Immunol 123(4):774-782 e775.

Schukken, Y. H., J. Gunther, J. Fitzpatrick, M. C. Fontaine, L. Goetze, O. Holst, J. Leigh, W. Petzl, H. J. Schuberth, A. Sipka, D. G. Smith, R. Quesnell, J. Watts, R. Yancey, H. Zerbe, A. Gurjar, R. N. Zadoks, H. M. Seyfert, and c. members of the Pfizer mastitis research. 2011. Host-response patterns of intramammary infections in dairy cows. Vet Immunol Immunopathol 144(3-4):270-289.

Schukken, Y. H., K. E. Leslie, D. A. Barnum, B. A. Mallard, J. H. Lumsden, P. C. Dick, G. H. Vessie, and M. E. Kehrli. 1999. Experimental Staphylococcus aureus intramammary challenge in late lactation dairy cows: quarter and cow effects determining the probability of infection. J. Dairy Sci. 82(11):2393-2401.

Selvamani, S. P., R. Mishra, and S. K. Singh. 2014. Chikungunya virus exploits miR-146a to regulate NF-kappaB pathway in human synovial fibroblasts. PLoS One 9(8):e103624.

Sharma, B. S., M. K. Abo-Ismail, F. S. Schenkel, Q. You, C. P. Verschoor, S. D. Pant, and N. A. Karrow. 2015. Association of TLR4 polymorphisms with Mycobacterium avium subspecies paratuberculosis infection status in Canadian Holsteins. Anim Genet 46(5):560-565.

Sheridan, M. P., G. Regev-Shoshani, J. Martins, S. Vimalanathan, and C. Miller. 2016. Nitric oxide modulates the immunological response of bovine PBMCs in an in vitro BRDc infection model. Res Vet Sci 109:21-28.

Shuster, D. E., E. K. Lee, and M. E. Kehrli, Jr. 1996. Bacterial growth, inflammatory cytokine production, and neutrophil recruitment during coliform mastitis in cows within ten days after calving, compared with cows at midlactation. Am J Vet Res 57(11):1569-1575.

Siebert, L., S. Headrick, M. Lewis, B. Gillespie, C. Young, L. Wojakiewicz, O. Kerro-Dego, M. E. Prado, R. Almeida, S. P. Oliver, and G. M. Pighetti. 2017. Genetic variation in CXCR1 haplotypes linked to severity of Streptococcus uberis infection in an experimental challenge model. Vet Immunol Immunopathol 190:45-52.

Simojoki, H., T. Orro, S. Taponen, and S. Pyorala. 2009. Host response in bovine mastitis experimentally induced with Staphylococcus chromogenes. Veterinary microbiology 134(1-2):95-99.

Sipka, A., A. Gurjar, S. Klaessig, G. E. Duhamel, A. Skidmore, J. Swinkels, P. Cox, and Y. Schukken. 2013. Prednisolone and cefapirin act synergistically in resolving experimental Escherichia coli mastitis. Journal of dairy science 96(7):4406-4418.

Sipka, A., B. Pomeroy, S. Klaessig, and Y. Schukken. 2016. Bovine natural killer cells are present in Escherichia coli infected mammary gland tissue and show antimicrobial activity in vitro. Comp Immunol Microbiol Infect Dis 48:54-60.

Smith, J. A., L. A. Samayawardhena, and A. W. Craig. 2010. Fps/Fes protein-tyrosine kinase regulates mast cell adhesion and migration downstream of Kit and beta1 integrin receptors. Cell Signal 22(3):427-436.

Sohn, E. J., M. J. Paape, E. E. Connor, D. D. Bannerman, R. H. Fetterer, and R. R. Peters. 2007. Bacterial lipopolysaccharide stimulates bovine neutrophil production of TNF-alpha, IL-1beta, IL-12 and IFN-gamma. Vet. Res. 38(6):809-818.

Song, M., Y. He, H. Zhou, Y. Zhang, X. Li, and Y. Yu. 2016. Combined analysis of DNA methylome and transcriptome reveal novel candidate genes with susceptibility to bovine Staphylococcus aureus subclinical mastitis. Sci Rep 6:29390.

Sordillo, L. M., G. M. Pighetti, and M. R. Davis. 1995. Enhanced production of bovine tumor necrosis factor-alpha during the periparturient period. Vet Immunol Immunopathol 49(3):263-270.

Spalluto, C. M., A. Singhania, D. Cellura, C. H. Woelk, T. Sanchez-Elsner, K. J. Staples, and T. M. A. Wilkinson. 2017. IFN-gamma Influences Epithelial Antiviral Responses via Histone Methylation of the RIG-I Promoter. Am J Respir Cell Mol Biol 57(4):428-438.

Spencer, S. J., E. Field, and Q. J. Pittman. 2010. Neonatal programming by neuroimmune challenge: effects on responses and tolerance to septic doses of lipopolysaccharide in adult male and female rats. J. Neuroendocrinol. 22(4):272-281.

Spencer, S. J., M. A. Galic, and Q. J. Pittman. 2011. Neonatal programming of innate immune function. Am. J. Physiol. Endocrinol. Metab. 300(1):E11-18.

Spencer, S. J., S. Martin, A. Mouihate, and Q. J. Pittman. 2006. Early-life immune challenge: defining a critical window for effects on adult responses to immune challenge. Neuropsychopharmacology 31(9):1910-1918.

Starkhammar, M., S. Kumlien Georen, S. E. Dahlen, L. O. Cardell, and M. Adner. 2015. TNFalpha-blockade stabilizes local airway hyperresponsiveness during TLR-induced exacerbations in murine model of asthma. Respir Res 16:129.

Steeneveld, W., H. Hogeveen, H. W. Barkema, J. van den Broek, and R. B. Huirne. 2008. The influence of cow factors on the incidence of clinical mastitis in dairy cows. Journal of dairy science 91(4):1391-1402.

Stein, M. M., C. L. Hrusch, J. Gozdz, C. Igartua, V. Pivniouk, S. E. Murray, J. G. Ledford, M. Marques Dos Santos, R. L. Anderson, N. Metwali, J. W. Neilson, R. M. Maier, J. A. Gilbert, M. Holbreich, P. S. Thorne, F. D. Martinez, E. von Mutius, D. Vercelli, C. Ober, and A. I. Sperling. 2016. Innate Immunity and Asthma Risk in Amish and Hutterite Farm Children. N Engl J Med 375(5):411-421.

Stevens, M. G., B. De Spiegeleer, L. Peelman, X. J. Boulougouris, A. V. Capuco, and C. Burvenich. 2012. Compromised neutrophil function and bovine E. coli mastitis: is C5a the missing link? Vet Immunol Immunopathol 149(3-4):151-156.

Stevens, M. G., L. J. Peelman, B. De Spiegeleer, A. Pezeshki, G. R. Van De Walle, L. Duchateau, and C. Burvenich. 2011. Differential gene expression of the toll-like receptor-4 cascade and neutrophil function in early- and mid-lactating dairy cows. Journal of dairy science 94(3):1277-1288.

Stifter, S. A. and C. G. Feng. 2015. Interfering with immunity: detrimental role of type I IFNs during infection. J Immunol 194(6):2455-2465.

Stojkovic, B., R. M. McLoughlin, and K. G. Meade. 2016. In vivo relevance of polymorphic Interleukin 8 promoter haplotype for the systemic immune response to LPS in Holstein-Friesian calves. Vet Immunol Immunopathol 182:1-10.

Stojkovic, B., M. P. Mullen, G. Donofrio, R. M. McLoughlin, and K. G. Meade. 2017. Interleukin 8 haplotypes drive divergent responses in uterine endometrial cells and are associated with somatic cell score in Holstein-Friesian cattle. Vet Immunol Immunopathol 184:18-28.

Stringhini, S., S. Polidoro, C. Sacerdote, R. S. Kelly, K. van Veldhoven, C. Agnoli, S. Grioni, R. Tumino, M. C. Giurdanella, S. Panico, A. Mattiello, D. Palli, G. Masala, V. Gallo, R. Castagne, F. Paccaud, G. Campanella, M. Chadeau-Hyam, and P. Vineis. 2015. Life-course socioeconomic status and DNA methylation of genes regulating inflammation. Int J Epidemiol 44(4):1320-1330.

Stringhini, S., P. Zaninotto, M. Kumari, M. Kivimaki, and G. D. Batty. 2016. Lifecourse socioeconomic status and type 2 diabetes: the role of chronic inflammation in the English Longitudinal Study of Ageing. Sci Rep 6:24780.

Sturdevant, G. L. and H. D. Caldwell. 2014. Innate immunity is sufficient for the clearance of Chlamydia trachomatis from the female mouse genital tract. Pathog Dis 72(1):70-73.

Suehiro, J., T. Hamakubo, T. Kodama, W. C. Aird, and T. Minami. 2010. Vascular endothelial growth factor activation of endothelial cells is mediated by early growth response-3. Blood 115(12):2520-2532.

Suojala, L., L. Kaartinen, and S. Pyorala. 2013. Treatment for bovine Escherichia coli mastitis - an evidence-based approach. J Vet Pharmacol Ther 36(6):521-531.

Takahashi, A., M. C. de Andres, K. Hashimoto, E. Itoi, and R. O. Oreffo. 2015. Epigenetic regulation of interleukin-8, an inflammatory chemokine, in osteoarthritis. Osteoarthritis Cartilage 23(11):1946-1954.

Takahashi, K., Y. Sugi, A. Hosono, and S. Kaminogawa. 2009. Epigenetic regulation of TLR4 gene expression in intestinal epithelial cells for the maintenance of intestinal homeostasis. J Immunol 183(10):6522-6529.

Tan, B. L., M. N. Yazicioglu, D. Ingram, J. McCarthy, J. Borneo, D. A. Williams, and R. Kapur. 2003. Genetic evidence for convergence of c-Kit- and alpha4 integrin-mediated

signals on class IA PI-3kinase and the Rac pathway in regulating integrin-directed migration in mast cells. Blood 101(12):4725-4732.

Taponen, S., E. Liski, A. M. Heikkila, and S. Pyorala. 2017. Factors associated with intramammary infection in dairy cows caused by coagulase-negative staphylococci, Staphylococcus aureus, Streptococcus uberis, Streptococcus dysgalactiae, Corynebacterium bovis, or Escherichia coli. Journal of dairy science 100(1):493-503.

Taraktsoglou, M., U. Szalabska, D. A. Magee, J. A. Browne, T. Sweeney, E. Gormley, and D. E. MacHugh. 2011. Transcriptional profiling of immune genes in bovine monocyte-derived macrophages exposed to bacterial antigens. Veterinary immunology and immunopathology 140(1-2):130-139.

Thakur, B. K., N. Dasgupta, A. Ta, and S. Das. 2016. Physiological TLR5 expression in the intestine is regulated by differential DNA binding of Sp1/Sp3 through simultaneous Sp1 dephosphorylation and Sp3 phosphorylation by two different PKC isoforms. Nucleic Acids Res 44(12):5658-5672.

Thompson-Crispi, K., H. Atalla, F. Miglior, and B. A. Mallard. 2014. Bovine mastitis: frontiers in immunogenetics. Frontiers in immunology 5:493.

Thorberg, B. M., M. L. Danielsson-Tham, U. Emanuelson, and K. Persson Waller. 2009. Bovine subclinical mastitis caused by different types of coagulase-negative staphylococci. Journal of dairy science 92(10):4962-4970.

Tiezzi, F., K. L. Parker-Gaddis, J. B. Cole, J. S. Clay, and C. Maltecca. 2015. A genome-wide association study for clinical mastitis in first parity US Holstein cows using single-step approach and genomic matrix re-weighting procedure. PLoS One 10(2):e0114919.

Uchiyama, R., B. Chassaing, B. Zhang, and A. T. Gewirtz. 2015. MyD88-mediated TLR signaling protects against acute rotavirus infection while inflammasome cytokines direct Ab response. Innate Immun 21(4):416-428.

Vakkamaki, J., S. Taponen, A. M. Heikkila, and S. Pyorala. 2017. Bacteriological etiology and treatment of mastitis in Finnish dairy herds. Acta Vet Scand 59(1):33.

van Delft, M. A., L. F. Huitema, and S. W. Tas. 2015. The contribution of NF-kappaB signalling to immune regulation and tolerance. Eur J Clin Invest 45(5):529-539.

van Eijk, K. R., S. de Jong, M. P. Boks, T. Langeveld, F. Colas, J. H. Veldink, C. G. de Kovel, E. Janson, E. Strengman, P. Langfelder, R. S. Kahn, L. H. van den Berg, S. Horvath, and R. A. Ophoff. 2012. Genetic analysis of DNA methylation and gene expression levels in whole blood of healthy human subjects. BMC Genomics 13:636.

Vanderhaeghen, W., S. Piepers, F. Leroy, E. Van Coillie, F. Haesebrouck, and S. De Vliegher. 2014. Invited review: effect, persistence, and virulence of coagulase-negative Staphylococcus species associated with ruminant udder health. Journal of dairy science 97(9):5275-5293.

Vangroenweghe, F., L. Duchateau, P. Boutet, P. Lekeux, P. Rainard, M. J. Paape, and C. Burvenich. 2005a. Effect of carprofen treatment following experimentally induced Escherichia coli mastitis in primiparous cows. Journal of dairy science 88(7):2361-2376.

Vangroenweghe, F., I. Lamote, and C. Burvenich. 2005b. Physiology of the periparturient period and its relation to severity of clinical mastitis. Domest Anim Endocrinol 29(2):283-293.

Vangroenweghe, F., P. Rainard, M. Paape, L. Duchateau, and C. Burvenich. 2004. Increase of Escherichia coli inoculum doses induces faster innate immune response in primiparous cows. Journal of dairy science 87(12):4132-4144.

Vasquez, A. K., D. V. Nydam, M. B. Capel, S. Eicker, and P. D. Virkler. 2017. Clinical outcome comparison of immediate blanket treatment versus a delayed pathogen-based treatment protocol for clinical mastitis in a New York dairy herd. Journal of dairy science 100(4):2992-3003.

Verbeke, J., S. Piepers, K. Supre, and S. De Vliegher. 2014. Pathogen-specific incidence rate of clinical mastitis in Flemish dairy herds, severity, and association with herd hygiene. Journal of dairy science 97(11):6926-6934.

Vu, T. H., T. Li, and A. R. Hoffman. 2004. Promoter-restricted histone code, not the differentially methylated DNA regions or antisense transcripts, marks the imprinting status of IGF2R in human and mouse. Hum Mol Genet 13(19):2233-2245.

Wang, B., Y. H. Rao, M. Inoue, R. Hao, C. H. Lai, D. Chen, S. L. McDonald, M. C. Choi, Q. Wang, M. L. Shinohara, and T. P. Yao. 2014. Microtubule acetylation amplifies p38 kinase signalling and anti-inflammatory IL-10 production. Nat Commun 5:3479.

Wang, X., P. Ma, J. Liu, Q. Zhang, Y. Zhang, X. Ding, L. Jiang, Y. Wang, Y. Zhang, D. Sun, S. Zhang, G. Su, and Y. Yu. 2015. Genome-wide association study in Chinese Holstein cows reveal two candidate genes for somatic cell score as an indicator for mastitis susceptibility. BMC Genet 16:111.

Wang, Z. W., P. Wang, F. H. Lin, X. L. Li, X. F. Li, K. T. O'Byrne, S. T. Hou, and R. Y. Zheng. 2011. Early-life exposure to lipopolysaccharide reduces the severity of experimental autoimmune encephalomyelitis in adulthood and correlated with increased urine corticosterone and apoptotic CD4+ T cells. Neuroscience 193:283-290.

Weaver, I. C., N. Cervoni, F. A. Champagne, A. C. D'Alessio, S. Sharma, J. R. Seckl, S. Dymov, M. Szyf, and M. J. Meaney. 2004. Epigenetic programming by maternal behavior. Nat Neurosci 7(8):847-854.

Weldon, A. J., I. Moldovan, M. G. Cabling, E. A. Hernandez, S. Hsu, J. Gonzalez, A. Parra, A. Benitez, N. Daoud, K. Colburn, and K. J. Payne. 2015. Surface APRIL Is Elevated on Myeloid Cells and Is Associated with Disease Activity in Patients with Rheumatoid Arthritis. J Rheumatol 42(5):749-759.

Wen, A. Y., K. M. Sakamoto, and L. S. Miller. 2010. The role of the transcription factor CREB in immune function. J Immunol 185(11):6413-6419.

Wenz, J. R., L. K. Fox, F. J. Muller, M. Rinaldi, R. Zeng, and D. D. Bannerman. 2010. Factors associated with concentrations of select cytokine and acute phase proteins in dairy cows with naturally occurring clinical mastitis. Journal of dairy science 93(6):2458-2470.

West, M. A. and W. Heagy. 2002. Endotoxin tolerance: A review. Critical care medicine 30(1 Supp):S64-S73.

Williams, C. L., J. L. Teeling, V. H. Perry, and T. P. Fleming. 2011. Mouse maternal systemic inflammation at the zygote stage causes blunted cytokine responsiveness in lipopolysaccharide-challenged adult offspring. BMC Biol 9:49.

Williams, D. L., C. F. Li, T. Z. Ha, T. Ozment-Skelton, J. H. Kalbfleisch, J. Preiszner, L. Brooks, K. Breuel, and J. B. Schweitzer. 2004. Modulation of the phosphoinositide innate resistance to polymicrobial 3-kinase pathway alters sepsis. Journal of Immunology 172(1):449-456.

Wilson, D. J., Y. T. Grohn, G. J. Bennett, R. N. Gonzalez, Y. H. Schukken, and J. Spatz. 2007. Comparison of J5 vaccinates and controls for incidence, etiologic agent, clinical severity, and survival in the herd following naturally occurring cases of clinical mastitis. Journal of dairy science 90(9):4282-4288.

Yang, F. L., C. Shen, B. X. He, Y. Y. Yang, D. C. Gong, and X. S. Li. 2015. The prevalence of heifer mastitis and its associated risk factors in Huanggang, Central China. Trop Anim Health Prod 47(1):87-92.

Yarilina, A., K. Xu, J. Chen, and L. B. Ivashkiv. 2011. TNF activates calcium-nuclear factor of activated T cells (NFAT)c1 signaling pathways in human macrophages. Proc Natl Acad Sci U S A 108(4):1573-1578.

Ye, R. D. and L. Sun. 2015. Emerging functions of serum amyloid A in inflammation. J Leukoc Biol 98(6):923-929.

Zanoni, I., R. Ostuni, G. Capuano, M. Collini, M. Caccia, A. E. Ronchi, M. Rocchetti, F. Mingozzi, M. Foti, G. Chirico, B. Costa, A. Zaza, P. Ricciardi-Castagnoli, and F. Granucci. 2009. CD14 regulates the dendritic cell life cycle after LPS exposure through NFAT activation. Nature 460(7252):264-268.

Zhang, W., X. Li, T. Xu, M. Ma, Y. Zhang, and M. Q. Gao. 2016. Inflammatory responses of stromal fibroblasts to inflammatory epithelial cells are involved in the pathogenesis of bovine mastitis. Exp Cell Res 349(1):45-52.

Zhang, X., R. Yang, Y. Jia, D. Cai, B. Zhou, X. Qu, H. Han, L. Xu, L. Wang, Y. Yao, and G. Yang. 2014. Hypermethylation of Sp1 binding site suppresses hypothalamic POMC in neonates and may contribute to metabolic disorders in adults: impact of maternal dietary CLAs. Diabetes 63(5):1475-1487.

Zhao, M., J. Wang, W. Liao, D. Li, M. Li, H. Wu, Y. Zhang, M. E. Gershwin, and Q. Lu. 2016. Increased 5-hydroxymethylcytosine in CD4(+) T cells in systemic lupus erythematosus. J Autoimmun 69:64-73.